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<b>(21) International Application Number:</b> PCT/US93/01705 <b>(22) International Filing Date:</b> 25 February 1993 (25.02.93)  <b>(30) Priority data:</b> 843,752 28 February 1992 (28.02.92) US  <b>(71) Applicant:</b> AUTOIMMUNE, INC. [US/US]; Reservoir Office Park, 822 Boylston Street, Chestnut Hill, MA 02167 (US).  <b>(72) Inventors:</b> WEINER, Howard ; 114 Somerset Road, Brookline, MA 02165 (US). MILLER, Ariel ; 24 Shimkin Street, Ahuza, 34 750 Haifa (IL). ZHANG, Zhengyi ; 31 Pheasant Street, Needham, MA 02192 (US). AL-SAB-BAGH, Ahmad ; 3403 Village Road West, Norwood, MA 02062 (US). HAFLER, David, Allen ; 110 Forest Avenue, West Newton, MA 02165 (US).		<b>(74) Agents:</b> FRANKFORT, Howard, M. et al.; Darby & Darby, 805 Third Avenue, New York, NY 10022 (US).  <b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>

**(54) Title:** BYSTANDER SUPPRESSION OF AUTOIMMUNE DISEASES

**(57) Abstract**

The invention is directed to a method for treating autoimmune diseases in mammals, the method comprising administering to the mammals an effective amount for treating the diseases of a bystander antigen, the antigen eliciting the release of transforming growth factor beta (TGF- $\beta$ ) at a locus within the body of the mammals wherein T-cells contributing to autoimmune response are found to suppress the T-cells contributing to the disease. The invention is directed also to compositions comprising bystander antigens useful in the treatment of autoimmune diseases in mammals and to pharmaceutical formulations thereof.

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<b>(54) Title:</b> BYSTANDER SUPPRESSION OF AUTOIMMUNE DISEASES  <b>(57) Abstract</b> <p>The invention is directed to a method for treating autoimmune diseases in mammals, the method comprising administering to the mammals an effective amount for treating the diseases of a bystander antigen, the antigen eliciting the release of transforming growth factor beta (TGF-<math>\beta</math>) at a locus within the body of the mammals wherein T-cells contributing to autoimmune response are found to suppress the T-cells contributing to the disease. The invention is directed also to compositions comprising bystander antigens useful in the treatment of autoimmune diseases in mammals and to pharmaceutical formulations thereof.</p>		

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10 BYSTANDER SUPPRESSION OF AUTOIMMUNE DISEASES

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This application is a C-I-P of:

Weiner et al., U.S. Patent Applications Serial Nos. 460,852 filed February 21, 1990, and 596,936, filed October 15, 1990 (the former being the national stage of PCT Application No. PCT/US88/02139, filed June 24, 1988), which in turn are continuation-in-part applications of U.S. Patent Application Serial No. 065,734 filed June 24, 1987;

Weiner et al., U.S. Patent Application Serial No. 454,486 filed December 20, 1989;

Weiner et al., U.S. Patent Application Serial No. 487,732, filed March 2, 1990;

Weiner et al., U.S. Patent Application Serial No. 551,632 filed July 10, 1990, in turn a Continuation-In-Part Application of U.S. Patent Application Serial No. 379,778, filed July 14, 1989 (now abandoned);

Weiner et al., U.S. Patent Application Serial No. 607,826 filed October 31, 1990; and

Weiner et al., U.S. Patent Application Serial No. 595,468, filed October 10, 1990.

FIELD OF THE INVENTION

This invention pertains to an improvement in the treatment of autoimmune diseases. More specifically, the invention is directed to the use of bystander antigens (i.e. antigens that suppress cells involved in the autoimmune process) for the treatment of autoimmune diseases. The invention also includes pharmaceutical formulations comprising bystander antigens useful in the treatment of autoimmune diseases in mammals.

10 BACKGROUND OF THE INVENTION

Autoimmune diseases are characterized by an abnormal immune response directed against normal autologous (self) tissues.

Based on the type of supranormal immune response involved, autoimmune diseases in mammals can generally be classified in one of two different categories: cell-mediated (i.e., T-cell-mediated) or antibody-mediated disorders. Non-limiting examples of cell-mediated autoimmune diseases include multiple sclerosis (MS), rheumatoid arthritis (RA), autoimmune thyroiditis (AT), diabetes mellitus (juvenile onset or Type 1 diabetes) and autoimmune uveoretinitis (AUR). Antibody-mediated autoimmune diseases include myasthenia gravis (MG) and systemic lupus erythematosus (SLE).

Both categories of autoimmune diseases are currently being treated with drugs which suppress immune responses in a non-specific manner, i.e., drugs which are incapable of suppressing selectively the abnormal immune response. Non-limiting examples of such drugs include methotrexate, cyclophosphamide, Imuran (azathioprine) and cyclosporin A. Steroid compounds such as prednisone and methylprednisolone (also non-specific immunosuppressants) are also employed in many instances. All of these currently employed drugs have limited efficacy against both cell- and antibody-mediated autoimmune diseases. Furthermore, such drugs have significant toxic and other side effects and, more important, eventually induce "global" immunosuppression in

the subject being treated. In other words, prolonged treatment with the drugs downregulates the normal protective immune response against pathogens thereby increasing the risk of infections. In addition, patients subjected to prolonged global  
5 immunosuppression have an increased risk of developing severe medical complications from the treatment, such as malignancies, kidney failure and diabetes.

In an effort to overcome the drawbacks of conventional treatments for autoimmune disease the present inventors and their  
10 coworkers have devised methods and pharmaceutical formulations useful for treating autoimmune diseases based on the concept of oral tolerization (or tolerization by inhalation) using as the tolerizers autoantigens, or disease-suppressive fragments or analogs of autoantigens alone or in combination with so-called  
15 "synergists", i.e., compounds which enhance the tolerizing effect of the autoantigens.

Autoantigens are antigens normally found within and specific for an organ or tissue under autoimmune attack which are themselves the primary target of autoimmune response.

20 Although the above methods and pharmaceutical formulations represent a substantial improvement in the treatment of autoimmune diseases, their therapeutic availability is delayed because in each case the specific autoantigens involved in eliciting and maintaining the disease state have to be identified. In other words, the specific substances that are the  
25 subject of attack by the immune system have to be determined. In many instances, this is both difficult and time-consuming, as those of ordinary skill in the art will appreciate. For example, more than one autoantigen may be the subject of autoimmune attack  
30 at any one time and the identity of the autoantigen(s) may change as the disease progressively destroys more and more of the tissue involved.

Therefore, what is needed in the art are improved agents, methods and compositions for treating individuals  
35 suffering from autoimmune diseases which would be more readily

available for therapeutic use, e.g., which would not require prior identification of autoantigens. There is also a need in the art for additional methods and compositions for treating autoimmune disease, which methods and compositions could be used  
5 in addition to or instead of autoantigens.

Furthermore, there is a need in the art for elucidating the mechanisms by which autoimmune disease can be combatted and for identifying novel methods and compositions in light of this newly acquired knowledge that can be used to combat autoimmune  
10 disease.

Accordingly, one object of the present invention is to provide improved methods and compositions for treating mammals suffering from autoimmune diseases, said methods and compositions to be used alone or optionally in combination with one or more  
15 autoantigens, synergists and other immune response regulators.

A further object of the present invention is to provide methods and compositions for treating mammals suffering from autoimmune diseases which can effectively be used to treat, alleviate the symptoms of, or prevent such diseases and do not  
20 require prior identification of the autoantigens involved in eliciting or maintaining the autoimmune disease.

Yet another object of the present invention is to provide methods and compositions for treating mammals afflicted with or susceptible to autoimmune diseases, which methods and  
25 compositions involve nontoxic agents, which are also, preferably, disease-specific.

#### SUMMARY OF THE INVENTION

The present invention is based on the unexpected and surprising discovery that oral or enteral administration (or  
30 administration by inhalation) of certain antigens (called "bystander antigens" and defined below) causes T-cells to be elicited that in turn suppress cells that contribute to immune attack of the organ or tissue involved in an autoimmune disease. The T-cells elicited by the bystander antigen mediate the release  
35 of transforming growth factor beta (TGF- $\beta$ ) which suppresses the

cells contributing to the immune attack that are found in the same vicinity.

For this type of suppression mechanism to work, it is not necessary that the TGF- $\beta$  releasing T-cells recognize the disease-contributing cells. All that is necessary is that both types of cells be found in the same vicinity when TGF- $\beta$  is released. One way to achieve this is to use as the bystander antigen an antigen that (a) has the ability to elicit T-cells that cause release of TGF- $\beta$  and (b) is itself specific to the tissue or organ under attack so that the suppressor T-cells that cause release of TGF- $\beta$  (and that are elicited pursuant to oral administration of the bystander antigen) will be directed to the same organ or tissue which is also a location where the disease-promoting cells are concentrated.

The bystander antigens may but do not need to be autoantigens, i.e. they do not need to be the same antigen(s) that is (are) under attack by the disease-inducing cells. It is an interesting feature of the present invention that oral administration of a bystander antigen can stave off tissue damage done by cells specific for another antigen or antigen fragment. This second antigen (or fragment) does not even need to have been identified.

Therefore, in one aspect the present invention is directed to a method for treating an autoimmune disease in a mammal, the method comprising administering to said mammal an effective amount for treating said disease of a bystander antigen, said antigen eliciting the release of transforming growth factor beta (TGF- $\beta$ ) at a locus within the body of said mammal wherein T cells contributing to autoimmune response are found to suppress the T-cells contributing to said response.

In another aspect, the present invention is directed to compositions and dosage forms comprising amounts of a bystander antigen effective to treat an autoimmune disease in a mammal.



In yet another aspect, the present invention provides a pharmaceutical inhalable dosage form for treating an autoimmune disease in a mammal, the form comprising an effective amount for treating said disease of a bystander antigen, said antigen upon administration eliciting the release of transforming growth factor beta (TGF- $\beta$ ) at a locus within the body of said mammal wherein T cells contributing to autoimmune response are found to suppress the T-cells contributing to said response; and a pharmaceutically acceptable carrier or diluent.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph showing the in vitro suppression of proliferative responses mediated by supernatants of lymphocytes or lymphocyte subsets isolated from orally tolerized animals.

15 Figure 2 is a bar graph showing the inhibition of in vitro suppression by anti-Transforming Growth Factor-beta (TGF- $\beta$ ) antibody.

Figure 3 is a bar graph showing TGF- $\beta$  activity in serum-free culture supernatants of suppressor T-cells isolated from orally tolerized animals.

Figure 4 (A-D) depicts a series of graphs showing the effects of anti-TGF- $\beta$  antibodies and control sera on experimental allergic encephalomyelitis (EAE) in orally tolerized (MBP-fed) and non-MBP-fed animals.

25 Figure 5 (A-D) depicts a series of bar graphs showing the effect of anti-TGF- $\beta$  antibodies on Delayed Type Hypersensitivity (DTH) responses in orally tolerized and control animals.

Figure 6 (A-C) depicts a series of graphs showing suppression of autoimmune disease associated with oral administration of a bystander antigen and substantially simultaneous immunization with MBP followed by injection of selected antigens.

Figure 7 is a bar graph showing Delayed Type Hypersensitivity (DTH) responses associated with such bystander suppression.

Figure 8 is a bar graph showing whether in vivo bystander suppression of EAE is associated with bovine serum albumin (BSA), ovalbumin (OVA) and myelin basic protein (MBP) fed animals immunized with MBP and injected with the same antigen as was fed.

Figure 9 is a bar graph showing that the adoptive transfer of bystander suppression is associated with CD8<sup>+</sup> suppressor T-cells.

Figure 10 is a bar graph showing the proliferation of T-cells (of the type which mediate EAE) in response to synthetic overlapping guinea pig MBP peptides by MBP-primed lymphoid cells.

Figure 11 is a bar graph showing the ability of various MBP peptides to trigger spleen cells from MBP-fed animals to suppress OVA-primed spleen cells in an in vitro transwell system.

Figure 12 (A) is a graph depicting the effect of feeding an autoantigen (PLP) or a bystander antigen (MBP) on EAE induced in SJL/J mice with a PLP-peptide; (B) is a bar graph summarizing the data of (A).

Figure 13 (A) is a graph depicting the tolerizing effect of intravenous administration of an autoantigen (PLP) or a bystander antigen (MBP) on EAE induced in SJL/J mice with PLP-peptide; (B) is a bar graph summarizing the data in (A).

Figure 14 is a bar graph showing the suppression of EAE (induced with MBP-peptide 71-90) by feeding various guinea pig MBP peptides alone or in combination with soybean trypsin inhibitor (STI).

Figure 15 (A-D) are a series of bar graphs showing DTH responses in animals immunized for EAE with either whole MBP or MBP peptide 71-90 and fed either whole MBP or various MBP peptides (alone or in combination with STI).

Figure 16 is a bar graph showing the effect of intravenous tolerization with MBP and disease inducing and non-inducing fragments thereof on induced EAE expression in SJL/J mice.

## 5 DETAILED DESCRIPTION OF THE INVENTION

All patent applications, patents, and literature references cited in this specification are hereby incorporated by reference in their entirety. In case of inconsistencies, the description including the definitions and interpretations of the  
10 present disclosure will prevail.

### Definitions

The following terms used in this disclosure shall have the meaning ascribed to them below:

(a) "Bystander antigen" or "bystander" is a protein,  
15 protein fragment, peptide, glycoprotein, or any other immunogenic substance (i.e. a substance capable of eliciting an immune response) that (i) upon oral or enteral administration (or administration by inhalation) elicits suppressor T-cells that cause TGF- $\beta$  to be released and thereby suppress cells that  
20 contribute to destruction of tissue during an autoimmune disease and even when the destructive cells are specific to a different immunogenic substance. Preferably, the suppressor T-cells elicited by the bystander will be targeted to the same tissue that is under attack during an autoimmune disease. The term  
25 therefore encompasses but is not limited to antigens capable of causing the foregoing release of TGF- $\beta$  and specific to the tissue or organ under attack in said autoimmune disease. The term also encompasses autoantigens and fragments or analogs thereof that have the ability to elicit such T-cell suppressors upon oral or  
30 enteral administration or upon inhalation. Thus, "bystander" is not coextensive with "autoantigen" as the latter is defined herein; an "autoantigen" is not also a "bystander" unless upon ingestion or inhalation it suppresses autoimmune response via the elicitation of T-suppressors that cause release of TGF- $\beta$  as  
35 described above.

(b) "Bystander suppression" is suppression of cells that contribute to autoimmune destruction by the release of the immunosuppressive factor TGF- $\beta$ , this release being in turn mediated by suppressor T-cells elicited by the ingestion or inhalation of a bystander antigen and recruited to the site where cells contributing to autoimmune destruction are found. The result is downregulation of the specific autoimmune response.

(c) "Mammal" is defined herein as any organism having an immune system and being susceptible to an autoimmune disease.

(d) "Autoimmune disease" is defined herein as a malfunction of the immune system of mammals, including humans, in which the immune system fails to distinguish between foreign substances within the mammal and/or autologous tissues or substances and, as a result, treats autologous tissues and substances as if they were foreign and mounts an immune response against them.

(e) "Autoantigen" is any substance or a portion thereof normally found within a mammal that, in an abnormal situation, is no longer recognized as part of the mammal itself by the lymphocytes or antibodies of that mammal, and is therefore the primary target of attack by the immunoregulatory system as though it were a foreign substance. The term also includes antigenic substances which induce conditions having the characteristics of an autoimmune disease when administered to mammals.

(f) "Treatment" is intended to include both the prophylactic treatment to prevent an autoimmune disease (or to prevent the manifestation of clinical or subclinical, e.g., histological symptoms thereof), as well as the therapeutic suppression or alleviation of symptoms after the onset of such autoimmune disease.

(g) "Synergists" are defined herein as substances which augment or enhance the suppression of the clinical (and/or histological) manifestation of autoimmune diseases when administered orally or by inhalation in conjunction with the adminis-

tration of a bystander antigen and/or an autoantigen. As used in the preceding sentence, and elsewhere in this specification, "in conjunction with" (also referred to herein as in association with) means before, substantially simultaneously with or after oral or aerosol administration of autoantigens and/or bystander antigens. Naturally, administration of the conjoined substance should not precede nor follow administration of the autoantigen or bystander antigen by so long an interval of time that the relevant effects of the substance administered first have worn off. Therefore, the synergists should be administered within about 24 hours before or after the autoantigen or bystander antigen, and preferably within about one hour.

(h) "Oral" administration includes oral, enteral or intragastric administration.

(i) A disease having the "characteristics" or "symptoms" of a particular autoimmune disease refers to a spontaneous or induced disease state that presents with specific inflammation of the same organ or tissue as that afflicted in the autoimmune disease. An example of an induced state is EAE, a model for multiple sclerosis. An example of a spontaneous state is diabetes developed by NOD mice.

#### Description of Bystander Suppression

It has now unexpectedly been discovered that the oral or by-inhalation administration of bystander antigens is an effective treatment for an autoimmune disease. At least cell-mediated autoimmune diseases can be treated using the methods and pharmaceutical formulations of the present invention.

Suppression mediated by oral administration of bystander antigens is brought about by elicitation of suppressor T-cells that release an immunosuppressive factor, transforming growth factor-beta (TGF- $\beta$ ). TGF- $\beta$  is not specific for the antigen triggering the suppressor cells that release it, even though these suppressor T-cells release TGF- $\beta$  only when triggered by the orally administered (or inhaled) antigen. Recruitment of the suppressor T-cells to a locus within a mammal where cells

contributing to the autoimmune destruction of an organ or tissue are concentrated allows for the release of TGF- $\beta$  in the vicinity of the disease-causing cells and suppresses (i.e. shuts down) these cells. The ability of TGF- $\beta$  to suppress these  
5 "destructive" cells is independent of the antigen for which the destructive cells may be specific.

The preferred way to accomplish suppression of the destructive cells is to select for oral administration to the mammal an antigen which is not only capable of eliciting  
10 suppressor T-cells capable of releasing TGF- $\beta$  but which is capable of targeting these suppressor T-cells to a location within the mammal's body where destructive cells are found in high concentration. The preferred and most efficient target for the suppressor T-cells is the organ or tissue under immune attack  
15 in the particular autoimmune disease involved because the destructive cells will be concentrated in the vicinity of that organ or tissue. Hence, it is preferred that the bystander antigen (to which the suppressor T-cells are specific) be itself an antigen specific to the tissue or organ under attack. Thus,  
20 the bystander antigen may be an autoantigen or preferably a non-disease inducing fragment or analog of an autoantigen (there is evidence that the parts or epitopes of autoantigens that are involved in inducing disease or in tissue destruction are not the same as those involved in bystander suppression; See Example 6  
25 below). More important, however, the bystander may be another antigen that is not an autoantigen; hence, the autoantigen (or autoantigens) involved need not be identified.

In more detail, the mechanism of bystander suppression according to the present invention for a tissue-specific  
30 bystander antigen is as follows: After a tissue-specific bystander antigen is administered orally (or enterally; i.e., directly into the stomach) or by inhalation, it passes into the small intestine, where it comes into contact with the so-called Peyer's Patches, which are collections of immunocytes located  
35 under the intestinal wall. These cells, are in turn in communi-

cation with the immune system, including the spleen and lymph nodes. The result is that suppressor (CD8+) T-cells are induced and recruited to the area of autoimmune attack, where they cause the release of TGF- $\beta$ , which can non-specifically downregulate the B-cells as well as the activated CD4+ T-cells directed against the mammal's own tissues. Despite the non-specific nature of the activity of TGF- $\beta$ , the resulting tolerance is specific for the autoimmune disease by virtue of the fact that the bystander antigen is specific for the tissue under attack and suppresses the immune cells that are found at or near the tissue being damaged.

Another instance of bystander suppression within the scope of the invention involves oral administration of an antigen that is neither an autoantigen nor specific to a tissue or organ under attack. To activate bystander suppression, injection with the same antigen has to take place. The ingested or inhaled antigen elicits formation of suppressor T-cells which are targeted to the microenvironment, pathway or inflamed tissue (depending on where the injected antigen localizes) where they cause the release of TGF- $\beta$ . Once released, TGF- $\beta$  suppresses all immune attack cells including the tissue-destructive cells.

#### TGF- $\beta$

TGF- $\beta$  affects cells of the immune system (e.g., T and B lymphocytes) thereby influencing inflammatory responses. T-lymphocytes (and other cells) produce TGF- $\beta$ ; it is released relatively late in the cascade of immune system response events (after T-cell activation) and is highly suppressive for both T- and B-cell proliferation. Numerous normal tissues have the ability to produce TGF- $\beta$ . These include human platelets, placenta, bovine kidney, bone, NK cells, B-cells, as well as CD4+ and CD8+ T-cells and activated macrophages. The isolation and biological properties of TGF- $\beta$  have been described in Transforming Growth Factor- $\beta$ s Chemistry, Biology, and Therapeutics, Piez, K.A. et al Eds, Ann. N.Y. Acad. Sci. 593:1-217, 1990.

Although TGF- $\beta$  was initially identified as a growth factor, it soon became clear that it was a substance having many and important immunoregulatory properties including inhibition of B- and T-cells and inhibition of the activity of CD4+ cells more than that of CD8+ cells, both in rodents and humans. TGF- $\beta$  is also known to antagonize inflammatory cytokines such as tumor necrosis factor (TNF) and gamma interferon (IFN- $\gamma$ ), block cytotoxic lymphocyte activity and inhibit the induction of receptors for Interleukin-1 (IL-1) and Interleukin-2 (IL-2) thereby rendering cells unresponsive to these cytokines. TGF- $\beta$  is a protein which has a molecular weight of 25 kD and is composed of two identical 12.5 kD subunits that are held together by a number of interchain disulfide bonds. At least two forms of TGF- $\beta$  exist: active and latent. Active TGF- $\beta$  has a short half-life and a small volume distribution whereas latent TGF- $\beta$  has an extended half-life and a larger volume distribution. Two isoforms of TGF- $\beta$  exist, TGF- $\beta$ 1 and TGF- $\beta$ 2. It is believed that TGF- $\beta$ 1 is involved in bystander suppression.

#### Animal Models

Throughout the present specification, reference is made to various model systems that have been developed for studying autoimmune diseases. Experimental autoimmune encephalomyelitis (EAE) has been studied in mice and other mammalian species as a model for Multiple Sclerosis (MS). Those of ordinary skill in the art recognize that virtually all potential immune therapies for MS are first tested in this animal model system. The disease is induced by parenteral administration of myelin basic protein (MBP) or proteolipid protein (PLP) and an adjuvant (such as Freund's Complete Adjuvant, FCA). This treatment, with either antigen, induces both a monophasic and an exacerbating/remitting form of demyelinating disease (depending on the species and details of administration). The induced disease has the characteristics of the autoimmune disease MS.

Parenteral administration of Mycobacterium tuberculosis with Freund's Complete Adjuvant in oil into the dorsal root tail



of susceptible mammals induces a disease with the characteristics of human rheumatoid arthritis. In like manner, parenteral administration of Type II collagen with an adjuvant will also induce a disease with the characteristics of human rheumatoid  
5 arthritis.

The administration to Lewis rats of S-antigen or IRBP-antigen with an adjuvant induces autoimmune uveoretinitis, whereas diabetes develops spontaneously in the NOD Mouse and the BB Rat.

10 One or more of the above disclosed model systems may be employed to demonstrate the efficacy and improved treatment provided by the present invention. In fact, the animal models are particularly suitable for testing therapies involving bystander suppression precisely because this mechanism allows  
15 suppression of all immune attack cells regardless of the antigen to which they are specific and is therefore unaffected by many of the actual or potential differences between a human autoimmune disorder and an animal model therefor.

The above animal models can be used to establish the  
20 utility of the present invention in mammals (including humans). For example, the present inventors orally administered a multiple sclerosis autoantigen, bovine myelin, to humans in a double-blind study and found that a certain patient subset received a considerable benefit from this treatment. In addition,  
25 rheumatoid arthritis symptoms, such as joint tenderness, AM stiffness, grip strength, etc., were successfully suppressed in humans receiving oral collagen (0.1-1.0 mg single dose daily). Finally, human trials with oral S-antigen showed very encouraging results for uveoretinitis. All of these human trials were  
30 attempted based on animal data using the appropriate disease model. Thus, the predictive value of animal models for therapeutic treatment of autoimmune diseases has been substantially enhanced.

#### Bystander Antigens

Bystander antigens not specific to the tissue under attack during autoimmune disease can be identified among nontoxic antigenic substances by using the same assay system as was used for OVA in e.g. Example 1.

5 Bystander antigens specific to a tissue or organ can be easily identified by testing the ability of such specific antigens to cause release of TGF- $\beta$ , which can be detected. For example, one or more potential tissue specific bystander antigens can be purified using well-known antigen purification techniques  
10 from an organ or tissue that is the target of autoimmune attack.

Bystander antigens and autoantigens (as well as fragments and analogs of any of them) can also be obtained using recombinant DNA technology, in bacterial, yeast, insect (e.g.  
15 psacalan virus) and mammalian cells using techniques well-known to those of ordinary skill in the art. Amino acid sequences for many potential and actual bystander antigens are known (disease-inducing epitopes should preferably not be used): See, e.g., Hunt, C. et al PNAS (USA), 82:6455-6459, 1985 (heat shock protein  
20 hsp70); Burkhardt, H., et al., Eur. J. Immunol. 21:49-54, 1991 (antigenic collagen II epitope); Tuohy, V.K., et al., J. Immunol. 142:1523-1527, 1989 (encephalitogenic determinant of mouse PLP); Shinohara, T. et al., In Progress in Retinal Research, Osborne, N. & Chader, J. Eds, Pergamon Press 1989, pp. 51-55 (S-antigen);  
25 Donoso, L.A., et al., J. Immunol. 143:79-83, 1989 (IRBP); Borst, D.E., et al., J. Biol. Chem. 264:115-1123, 1989 (IRBP); Yamaki, K. et al., FEBS 234:39-43, 1988 (S-antigen); Donoso, L.A. et al., Eye Res. 7:1087, 1988 (IRBP); Wyborski, R.J., et al., Mol. Brain Res. 8:193-198, 1990 (GAD).

30 The amino acid sequences for bovine PLP; bovine, human, chimpanzee, rat, mouse, pig, rabbit, guinea pig MBP; human and bovine collagen alpha-1(II) and bovine collagen alpha-1(I); and human insulin although taken from published sources, are provided in Appendix A for convenience.

In addition, some tissue-specific antigens are commercially available: e.g. insulin, glucagon, myelin basic protein, collagen I, collagen II, etc.

The potential bystander can then be fed to mammals and  
5 spleen cells or circulating T-cells from, e.g. the blood or  
cerebrospinal fluid in the case of EAE or MS, from these mammals  
can be removed, and stimulated in vitro with the same antigen.  
T-cells elicited by stimulation can be purified and supernatants  
can be tested for TGF- $\beta$  content quantitatively and/or  
10 qualitatively using e.g. a suitable commercially available  
polyclonal or preferably monoclonal antibody raised against TGF- $\beta$   
or another known assay for TGF- $\beta$  detection such as that described  
in Example 1 below using a commercially available mink lung  
epithelial cell line. Such methods for testing for TGF- $\beta$  are  
15 described in detail in the Examples, below. Methods for  
ascertaining the bystander potential of peptides derived from  
autoantigens are also illustrated in the Examples.

#### Use of Bystander Antigens - Dosages

The tolerance induced by the bystander antigens of this  
20 invention is dose-dependent over a broad range of oral (or  
enteral) or inhalable dosages. However, there are minimum and  
maximum effective dosages. In other words, suppression of the  
clinical and histological symptoms of an autoimmune disease  
occurs within a specific dosage range which however varies from  
25 disease to disease, mammal to mammal and bystander antigen to  
bystander antigen. For example, when the disease is PLP- or MBP-  
induced EAE in mice, the suppressive dosage range when MBP is  
used as the bystander is from about 0.1 to about 1  
mg/mouse/feeding (with feedings occurring about every other day  
30 (e.g., 5-7 feedings over a 10-14-day period). A most preferred  
dosage is 0.25 mg/mouse/feeding. For suppression of the same  
disease in rats, the MBP suppressive dosage range is from about  
0.5 to about 5 mg/rat/feeding and the most preferred dosage is 1  
mg/rat/feeding. The effective dosage range for humans with MS,  
35 when MBP is used, is between about 1 and about 100, preferably

between about 1 and about 50 mg MBP per day (administered every day or on alternate days) with the optimum being about 30 mg/day.

For rheumatoid arthritis, the effective dosage range for humans receiving either Type I or II collagen is about 0.1 to about 1 mg/day. For adjuvant-induced arthritis in mice the effective collagen dosage range is about 3 to about 30 micrograms/feeding with the same feeding schedule as for EAE.

Ascertaining the effective dosage range as well as the optimum amount is well within the skill in the art. For example, dosages for mammals and human dosages can be determined by beginning with a relatively low dose (e.g., 1 microgram), progressively increasing it (e.g. logarithmically) and measuring the amount of TGF-beta in the blood and/or scoring the disease severity, according to well-known scoring methods (e.g., on a scale of 1 to 5, or by measuring the number of attacks, or by measuring joint swelling, grip strength, stiffness, vision, etc. depending on the type of disease). The optimum dosage will be the one generating the maximum amount of TGF-beta in the blood and/or cause the greatest decrease in disease symptoms. An effective dosage range will be one that causes at least a statistically significant attenuation of at least one symptom characteristic of the disease being treated.

The present invention can also be advantageously used to prevent the onset of an autoimmune disease in susceptible individuals at risk for an autoimmune disease. For example, methods for the identification of patients who are at risk for developing Type 1 diabetes are extant and reliable and have been recently endorsed by the American Diabetes Association (ADA). Various assay systems have been developed which (especially in combination) have a high predictive value assessing susceptibility to Type 1 diabetes (Diabetes Care 13: 762-775, 1990. Details of one preferred screening test are available to those of ordinary skill in the art (Bonifacio, E. et al., The Lancet 335: 147-149, 1990).

From a practical point of view, preventing the onset of most autoimmune diseases is not as important a measure as it is in the case of diabetes. MS, RA, AT and AUR are declared at an early stage, before substantial tissue damage has taken place; therefore preventive treatment of these diseases is not as important as in the case of diabetes.

A non-limiting list of autoimmune diseases and tissue- or organ-specific confirmed or potential bystander antigens effective in the treatment of these diseases when administered in an oral or inhalable form are set forth in Table 1 below. Administration of combinations of antigens listed for each individual disease is also expected to be effective in treating the disease.

TABLE 1	
<u>Autoimmune Disease</u>	<u>Bystander Antigen</u>
5 <u>Type 1 Diabetes</u> (While beta-cell function is still present)	Glucagon, insulin, GAD (gamma amino decarboxylase), heat shock protein
<u>Multiple Sclerosis</u>	MBP, MBP fragments (especially non-inducing), PLP, PLP fragments (especially non-inducing)
<u>Rheumatoid Arthritis</u>	Collagen, collagen fragments (especially non-inducing), heat shock protein
<u>Uveoretinitis</u>	S-antigen, S-antigen fragments (especially non-inducing), IRBP (Interphotoreceptor Retinoid Binding Protein) and fragments thereof (especially non-inducing)

10 For any autoimmune disease, tissue extracts can be used as well as specific bystander antigens. For example, myelin has been used for MS and pancreatic extracts have been used for Type 1 diabetes. However, administration of one or more individual antigens is preferred.

15 Thus, according to the present invention, when treating Type 1 diabetes, an effective amount (determined as described above) of glucagon can be administered orally or by inhalation. Glucagon is specifically present in the pancreas. Glucagon, however, is not an autoantigen because it is pancreatic beta  
20 cells that are destroyed in the course of Type 1 diabetes whereas glucagon is found exclusively in alpha cells, a different cell type. Thus, glucagon is a "pure" bystander: it does not have any autoantigen activity.

25 Insulin definitely has bystander activity for Type 1 diabetes. It is not at present known whether insulin is also an autoantigen. However, whatever the mechanism of action, oral, enteral or inhalable insulin preparations are effective in suppressing diseases with the characteristics of Type 1 diabetes

as per. copending commonly assigned patent application Serial No. 595,468.

For diseases having the characteristics of multiple sclerosis, non-inducing fragments of MBP, e.g. a peptide  
5 comprising guinea pig MBP amino acids 21-40 act as bystanders not only for MBP-induced diseases (i.e. when MBP is the primary target of autoimmune attack) but also for PLP-induced disease (when PLP is the primary target of autoimmune attack).

For rheumatoid arthritis and animal models therefor,  
10 Type-I and Type-II collagen have bystander activity.

For diseases having the characteristics of uveoretinitis, S-antigen has bystander activity.

Noninducing fragments of those bystander antigens that are also autoantigens are preferred. Such fragments can be  
15 determined using the overlapping peptide method of Example 3 (which is a general technique although in Example 3 it is described specifically with respect to identification of noninducing fragments of MBP).

The present inventors have also discovered that orally  
20 administered autoantigens and bystander antigens both possess epitopes which specifically induce the production and/or release of TGF- $\beta$ . Although immunodominant epitopes of e.g., MBP have previously been disclosed, i.e., those epitopes which a majority of patients' CD4+ T lymphocytes recognize and proliferate in  
25 response to, or which a majority of a patient's antibodies recognize, immunosuppressive epitopes, i.e., those that elicit the production and/or release of TGF- $\beta$ , have not been disclosed or suggested before the present invention. Therefore, oral or by-inhalation administration of peptides encompassing these  
30 epitopes is expected to be more specific in eliciting bystander suppression than administration of the entire antigen without the risk of sensitizing the animal to disease-inducing or disease-propagating portions of an autoantigen. The immunosuppressive epitopes can be identified using the method described in Example

3 for the identification of MBP-peptide 21-40. (See also Fig.14.)

The bystander antigens can be administered in conjunction with autoantigens (the combination being effective) to treat or prevent autoimmune diseases. Autoantigen administration is carried out as disclosed in U.S. Patent Application Serial Nos. 460,852, 596,936, 454,486, 551,632, 502,559, 607,826 and 595,468 mentioned above. It is anticipated that co-administration of specific autoantigens (and preferably non-inducing fragments of autoantigens) with other bystander antigens will provide effective suppression of the autoimmune diseases.

In addition, synergists can be conjoined in the treatment to enhance the effectiveness of the above. Non-limiting examples of synergists for use in the present invention include bacterial lipopolysaccharides from a wide variety of gram negative bacteria such as various subtypes of E. coli and Salmonella (LPS, Sigma Chemical Co., St. Louis, MO; Difco, Detroit, MI; BIOMOL Res. Labs., Plymouth, PA), Lipid A (Sigma Chemical Co., St. Louis, MO; ICN Biochemicals, Cleveland, OH; Polysciences, Inc., Warrington, PA) and immunoregulatory lipoproteins, such as peptides covalently linked to tripalmitoyl-S-glycerylcysteinyl-seryl-serine (P<sub>3</sub> C55) which can be obtained as disclosed in Deres, K. et al. (Nature, 342:561-564, 1989) or "Brauns" lipoprotein from E. coli which can be obtained as disclosed in Braun, V., Biochim. Biophys. Acta 435:335-337, 1976. LPS is preferred and Lipid A particularly preferred. Lipid A is particularly preferred for use in the present invention because it is less toxic than the entire LPS molecule. LPS for use in the present invention can be extracted from gram-negative bacteria and purified using the method of Galanes et al. (Eur. J. Biochem. 9:245, 1969) and Skelly, R.R., et al. (Infect. Immun. 23:287, 1979).



Formulations

In another aspect, the present invention also provides oral pharmaceutical formulations for treating mammals suffering from autoimmune diseases comprising an amount of a bystander antigen (as described below) effective to suppress the autoimmune disease. The formulations optionally further comprise a synergist as disclosed in copending U.S. Patent Application, Serial No. 487,732, filed March 2, 1990 in an amount effective (in conjunction with the bystander antigen of the present invention) to treat the clinical symptoms of specific autoimmune diseases. Synergists, when administered in conjunction with bystander antigens, cause an increase of cytokines PGE (prostaglandin-E) and IL-4 (interleukin-4) in the vicinity of the target organ.

Throughout this discussion, it will be understood that any statistically significant attenuation of even one symptom of an autoimmune disease pursuant to the treatment of the present invention is within the scope of the invention.

Each oral (or enteral) formulation according to the present invention may additionally comprise inert constituents including pharmaceutically acceptable carriers, diluents, fillers, solubilizing or emulsifying agents, and salts, as is well-known in the art. For example, tablets may be formulated in accordance with conventional procedures employing solid carriers well-known in the art. Capsules employed in the present invention may be made from any pharmaceutically acceptable material, such as gelatin, or cellulose derivatives. Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are also contemplated, such as those described in U.S. Patent No. 4,704,295, issued November 3, 1987; U.S. Patent No. 4,556,552, issued December 3, 1985; U.S. Patent No. 4,309,404, issued January 5, 1982; and U.S. Patent No. 4,309,406, issued January 5, 1982.

Examples of solid carriers include starch, sugar, bentonite, silica, and other commonly used carriers. Further

non-limiting examples of carriers and diluents which may be used in the formulations of the present invention include saline, syrup, dextrose, and water.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount, since the necessary effective amount can be reached by administration of a plurality of dosage units (such as capsules or tablets or combinations thereof).

The route of administration of the bystander antigens of the present invention is preferably oral or enteral. The preferred oral or enteral pharmaceutical formulations may comprise, for example, a pill or capsule containing an effective amount of one or more of the bystander antigens of the present invention with or without an effective amount of a synergist.

In general, when administered orally or enterally, the bystander antigen may be administered in single dosage form or multiple dosage forms.

The effective amount of a synergist, e.g. LPS or Lipid A, to be administered in conjunction with the bystander broadly ranges between about 0.15 and 15 mg per kg body weight of said mammal per day and preferably between about 0.3 and 12 mg per kg body weight of said mammal per day.

In an alternative preferred embodiment of the present invention the pharmaceutical formulations or dosage forms of the present invention can also be administered to mammals suffering from autoimmune diseases by inhalation, preferably in aerosol form. The inhalation mode of administration is preferably not through the nasal passages but through the bronchial and pulmonary mucosa. It is expected that lower amounts of the bystander antigens of the present invention will be required using aerosol administration for treating an autoimmune disease as it has been found when treating experimental autoimmune encephalomyelitis (EAE) with myelin basic protein (MBP) and adjuvant arthritis with collagen as disclosed in co-pending U.S.

Patent Application Serial No. 454,486 filed December 20, 1989. The amounts of the bystander antigens of the present invention which may be administered in an aerosol dosage form would be between about 0.1 mg and about 15 mg per kg body weight of a mammal per day and may optionally include a synergist in amounts ranging between about 0.1 and about 15 mg per kg body weight of said mammal per day and may be administered in single dosage form or multiple dosage forms. The exact amount to be administered will vary depending on the state and severity of a patient's disease and the physical condition of the patient.

The aerosol pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing and emulsifying agents, and salts of the type that are well-known in the art. Examples of such substances include normal saline solutions, such as physiologically buffered saline solutions, and water.

The route of administration of the bystander antigens according to this alternate embodiment of the present invention is in an aerosol or inhaled form. The bystander antigens and related compounds of the present invention can be administered as dry powder particles or as an atomized aqueous solution suspended in a carrier gas (e.g. air or N<sub>2</sub>). Preferred aerosol pharmaceutical formulations may comprise for example, a physiologically-acceptable buffered saline solution containing between about 1 mg and about 300 mg of the bystander antigens of the present invention.

Dry aerosol in the form of finely divided solid particles of bystander antigens that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. The bystander antigens may be in the form of dusting powders and comprise finely divided particles having an average particle size of between about 1 and 5 microns, preferably between 2 and 3 microns. Finely divided particles may be prepared by pulverization and screen filtration using techniques well known in the art. The particles may be administered by

inhaling a predetermined quantity of the finely divided material, which can be in the form of a powder.

Specific non-limiting examples of the carriers and/or diluents that are useful in the aerosol pharmaceutical formulations of the present invention include water and physiologically-acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0. Additional non-limiting examples of suitable carriers or diluents for use in the aerosol pharmaceutical formulations or dosage forms of the present invention are disclosed in U.S. Patent Nos. 4,659,696, issued April 21, 1987, 4,863,720, issued September 5, 1989 and 4,698,332, issued October 6, 1987.

The pharmaceutical formulations of the present invention may be administered in the form of an aerosol spray using for example, a nebulizer such as those described in U.S. Patent Nos. 4,624,251 issued November 25, 1986; 3,703,173 issued November 21, 1972; 3,561,444 issued February 9, 1971 and 4,635,627 issued January 13, 1971. The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S.P. in Aerosols and the Lung, Clarke, S.W. and Davia, D. eds. pp. 197-224, Butterworths, London, England, 1984, can be used when practicing the present invention.

Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, MA), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co. (Valencia, CA).

As will be understood by those skilled in the art, the exact dosage and frequency of administration of the bystander antigens of the present invention (in oral or aerosol form) is a function of the activity of the bystander antigen, as well as the age, sex, weight, and physical condition of the subject to be treated, and the concurrent administration or absence of other treatments. Consequently, adjustment of the dosages used and

administration schedules must be determined based on these factors, and may need to be determined experimentally. Such determination, however, requires no more than routine experimentation, given the guidelines contained herein.

5 Experimental

In the examples below, which are intended to illustrate the present invention without limiting its scope the following are demonstrated:

Example 1 shows that the active form of TGF- $\beta$ 1 isotype  
10 mediates suppression of CD4<sup>+</sup> T-cells specific to MBP and that CD8<sup>+</sup> T-cells induced by feeding MBP to animals cause the release of TGF- $\beta$  and that it is TGF- $\beta$  that is responsible for suppression. The same example also demonstrates that antigens that are not autoantigens and that are not even specific to the  
15 tissues or organs under autoimmune attack can elicit formation of T-suppressor cells which cause the release of TGF- $\beta$ . This is illustrated by the oral administration of ovalbumin. The problem with ovalbumin, however, is that since it is not specific to the autoimmune afflicted tissue, it is by itself incapable of  
20 targeting the T-suppressor cells to a site where cells contributing to autoimmune attack can be found. (This problem is addressed in Example 2.) Example 1 also illustrates that not every orally administered antigen causes bystander-type suppression: bovine serum albumin does not.

25 Finally, Example 1 also demonstrates that the same mechanism (bystander suppression) is at work in suppression of EAE by oral administration of MBP.

Example 2 shows that an antigen capable of bystander suppression will upon oral administration cause the release of  
30 TGF- $\beta$  and that, furthermore, if the suppressor T-cells elicited by this antigen can be recruited to a location where cells contributing to autoimmune attack can be found, those disease-promoting cells will be suppressed. Example 2 further provides a way to effect such recruitment even when the antigen is not  
35 specific to the tissue under autoimmune attack. Finally, Example

2 shows that the suppressor T-cells that allow for the release of TGF- $\beta$  do not have to encounter the suppressed cells in order for suppression to take place.

The way a non-specific bystander antigen can be rendered an efficient bystander (i.e. "forced" to cause TGF- $\beta$  to be released in the vicinity of the disease-promoting cells) is by substantially simultaneous injection with the same antigen (within 24 hours before or after bystander oral administration). For example, when OVA was fed to animals and then these animals were immunized with MBP/CFA to induce EAE, it was found that an injection with OVA would suppress EAE. This is due to the concentration of both EAE promoting cells (which the OVA-elicited suppressor T-cells do not recognize) and cells specific to OVA (which are specific to OVA, just as the OVA-elicited suppressor T-cells) in the lymph nodes of the animal. The implication of this showing for therapy is that non-specific bystander antigens could also be used in combatting autoimmune disease if their suppressor T-cells can be targeted to a site where they would suppress disease-promoting cells. Although use of such non-specific bystander antigens is envisioned primarily as an adjuvant to specific bystander therapy, it is clearly within the scope of the invention.

Example 3 illustrates a technique for identifying noninducing fragments and, more important, immunosuppressive epitopes for one bystander which is also an autoantigen: MBP. However, the technique is general and can be applied to any bystander antigen as long as its amino acid sequence is known. (See also Figs 10, 11 and 14.)

Example 3 also indicates that there are portions of autoantigens (for example, the immunodominant epitope of guinea pig MBP, MBP 71-90, which is an EAE-inducing fragment) which do not participate in triggering TGF- $\beta$  release, and do not participate in bystander suppression. Thus, Example 3 illustrates that "pure" bystander suppression is desired, at least the immunodominant (disease-inducing) portions of an

autoantigen should not be used and that constructs should be made instead including the immunosuppressive epitopes and excluding the disease-promoting epitopes. Example 3 also shows that there are immunosuppressive epitopes in antigens capable of bystander suppression and that in the case of autoantigens, the immunosuppressive epitopes are different from those responsible for autoimmune response

Example 4 demonstrates that the types of cells and cytokines involved in autoimmune response and its suppression indeed are present (or absent) in the cortices and cerebella of naive (control) animals or those immunized with MBP/CFA and/or fed MBP, or fed MBP and the synergist LPS.

Example 5 illustrates the efficacy of insulin A-chain, insulin B-chain and of each of four insulin, B-chain fragments as well as glucagon in bystander suppression of insulinitis associated with Type 1 diabetes (NOD model). Insulinitis, an inflammatory response observed in the islet cells provides a good marker for gauging the efficacy of Type 1 diabetes autoimmune suppression because insulinitis (a) is triggered by the same mechanism as Type 1 diabetes and (b) persists only while autoimmune destruction is still taking place, i.e. while the subject maintains at least some islet cell function.

Example 6 demonstrates that one autoantigen can act solely as a bystander for another autoantigen. MBP was thus demonstrated to be a bystander for PLP. (PLP also has the ability of suppressing MBP-induced disease and therefore PLP is a bystander for MBP.)

Example 6 also shows that unlike bystander suppression, I.V. tolerization requires that the same antigen be administered as that which is the target of autoimmune attack (or which induces the disease, in an animal model).

**EXAMPLE 1:** Suppressor T-Cells Generated By Oral Tolerization To Myelin Basic Protein Suppress Both In Vitro And In Vivo Immune Responses By The Release Of TGF- $\beta$  Following Antigen Specific Triggering

In the experiments described below the following materials and methods were used.

Animals. Female Lewis rats 6-8 weeks of age were obtained from Harlan-Sprague Dawley Inc. (Indianapolis, IN).  
5 Animals were maintained on standard laboratory chow and water ad libitum.

Antigens. Guinea pig myelin basic protein (MBP) was purified from brain tissue by the modified method of Deibler et al. (Prep.Biochem. 2:139,1972) as disclosed in U.S. Patent  
10 Application Serial No. 07/487,732 filed March 2, 1990. Protein content and purity were checked by gel electrophoresis and amino acid analysis..

Reagents. Commercial reagents used were as follows: monoclonal mouse anti-rat IFN $\gamma$  neutralizing antibody (Amgen  
15 Biologicals, Thousand Oaks, CA); monoclonal hamster anti-murine TNF $\alpha$ + $\beta$  antibody (Genzyme, Boston, MA); polyclonal rabbit anti-TGF- $\beta_{1+2}$  neutralizing antibody (R & D Systems, Inc., Minneapolis, MN), and indomethacin (Sigma, St. Louis, MO). Turkey antiserum specific for the type 1 isoform of TGF- $\beta$  was prepared as  
20 previously described (Danielpour, D., et al. J. Cell. Physiol. 138: 79-86,1989).

Induction Of Oral Tolerance. Rats were fed 1 mg of MBP dissolved in 1 ml PBS, or PBS alone, by gastric intubation using a 18-gauge stainless steel animal feeding needle (Thomas  
25 Scientific, Swedesboro, NJ). Animals were fed five times at intervals of 2-3 days with the last feeding two days before immunization. The purpose of this was to induce tolerance.

In Vitro Suppression Of Proliferative Responses By Supernatants. Spleen cells were removed 7-14 days after the last  
30 feeding and a single cell suspension prepared by pressing the spleens through a stainless steel mesh. For preparation of supernatants, spleen cells at a concentration of  $5 \times 10^6$  cells/ml were stimulated in vitro with MBP (50  $\mu$ g/ml) in 10 ml of proliferation medium. Proliferation medium consisted of RPMI  
35 1640 (GIBCO, Grand Island, NY) supplemented with  $2 \times 10^{-5}$  M 2-



mercaptoethanol, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% glutamine, 1% HEPES buffer, 1% nonessential amino acids and 1% autologous serum. Supernatants were harvested at 24 hours and 100  $\mu$ l added to  $2.5 \times 10^4$  MBP specific T-cells, raised and maintained as previously described (Ben-Nun, A. et al., Eur. J. Immunol. 11:195-199, 1981), cultured with  $5 \times 10^5$  irradiated (2500 rad) thymocytes, in 100  $\mu$ l of proliferation media. MBP (50  $\mu$ l/ml) was added to the culture in a volume of 20  $\mu$ l. Experiments were performed in triplicate in round bottomed 96-well plates (Costar, Cambridge, MA). Cells were cultured for 72 hours at 37°C in an incubator with humidified 6% CO<sub>2</sub> and 94% air atmosphere, and each well was pulsed with 1  $\mu$ Ci of <sup>3</sup>H thymidine for the last 18 hours of culture. Cultures were harvested onto fiberglass filters using a multiharvester and counted using standard liquid scintillation techniques.

The purpose of this was to set up an assay system for soluble factors produced in oral tolerization.

Purification Of T-Cell Subsets. Depletion of lymphocyte subsets was performed by negative selection using magnetic beads according to a modified method of Cruikshank (J. Immunol. 138: 3817-3823, 1987). Spleen cells were incubated with a 1:10 dilution of mouse anti-rat CD8, CD4, or B-cell monoclonal antibodies (mAbs) (clones OX/8, W3/25 or OX/33 respectively, commercially available from Serotec/Bioproducts, Indianapolis, IN) for 30 minutes on ice, washed twice, and then added to prewashed magnetic particles, with an average diameter of 4.5  $\mu$ m (M-450) with goat anti-mouse IgG covalently attached (Dynal Inc., Fort Lee, NJ). The quantity of magnetic beads used was calculated as being 10 times the estimated target cell population. The cells were incubated with the beads in 0.5 ml of RPMI 1640 supplemented with 10% fetal calf serum (FCS) in a 10 ml round bottomed test tube (Nunc) for 30 minutes on ice with gentle shaking every 5 minutes. After incubation, the bead/cell suspension was washed with 5 ml of medium and the cell-mAb-bead complexes were separated from unlabelled cells in a strong

magnetic field using a magnetic-particle concentrator (DynaI-MPC-1) for two minutes. The supernatant was removed, and the procedure repeated twice to obtain the nonadherent fraction. The cells in the T-cell and B-cell depleted populations were >95% CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup>CD8<sup>+</sup> or CD4<sup>+</sup>CD8<sup>+</sup>OX/33<sup>-</sup> (B-cell depleted) as demonstrated by indirect flow cytometry. Whole spleen populations (5 x 10<sup>6</sup> cells) from MBP fed or control fed animals were cultured in the presence of MBP (50 µg/ml) in 1 ml of serum-free proliferation media. Depleted populations were cultured at a concentration of 2.5 x 10<sup>6</sup> cells/ml. Supernatants were collected at 24 hours and 100 µl added to responder cells as described above.

The purpose of this was to isolate specific subsets of T-cells in order to determine which T-cells were involved in Bystander Suppression.

Treatment Of Supernatants With Anti-Cytokine Antibodies. Spleen cells (5 x 10<sup>6</sup>/ml in proliferation media) from MBP-fed and control animals were incubated in the presence of MBP (50 µg/ml) plus neutralizing antibodies against interferon-gamma (INFγ), TGF-β, Tumor Necrosis Factor (TNF)α+β or with indomethacin for 72 hours. Antibodies were tested in a range of concentrations (1:250, 1:500, 1:1000) and indomethacin tested at concentrations of 0.5-1 µg/ml. At 24 hours, supernatants were collected and free antibody or antibody-cytokine complexes were removed using magnetizable polymer beads (Dynabeads, Dynal, Inc., Fort Lee, NJ). Beads coupled with anti-immunoglobulin antibodies were incubated at a concentration of 4 x 10<sup>7</sup> beads/ml for 30 minutes (done twice for each sample) and removed according to a modified method of Liabakk et al. (Scand. J. Immunol. 30:641, 1989), using a Dynal Magnetic Particle Concentrator (Dynal, MPC-1).

The purpose of these experiments was to examine the soluble cytokines produced upon oral tolerization.

Measurement Of TGF-β Activity In Serum-Free Culture Supernatants. Serum free culture supernatants were collected as

previously described (Kehri, et al. J. Exp. Med. 163: 1037-1050, 1986; Wahl, et al. J. Immunol. 145: 2514-2419, 1990). Briefly, modulator cells were first cultured for 8 hours with MBP (50  $\mu$ l/ml) in proliferation medium. Thereafter cells were washed  
5 three times and resuspended in serum-free medium for the remainder of the 72 hour culture, collected, then frozen until assayed. Determination of TGF- $\beta$  content and isoform type in supernatants was performed using a mink lung epithelial cell line (American Type Culture Collection, Bethesda, MD #CCL-64) according to  
10 Danielpour et al. (supra), and confirmed by a Sandwich Enzyme Linked Immunosorbent Assay (SELISA) assay as previously described (Danielpour et al. Growth Factors 2: 61-71, 1989). The percent active TGF- $\beta$  was determined by assay without prior acid activation of the samples.

15 The purpose of these experiments was to measure and determine the isoform of the TGF- $\beta$  produced by T-cells obtained from orally tolerized animals.

Immunization Of Animals. To induce a substantial EAC disease state, Lewis rats were immunized with 25  $\mu$ g of MBP in 50  
20  $\mu$ l in the left food pad, emulsified in an equal volume of complete Freund's adjuvant containing 4 mg/ml of Mycobacterium tuberculosis (Difco).

In Vivo Administration Of Anti-TGF- $\beta$  Antiserum And Control Sera. Turkey anti-TGF- $\beta$  antiserum specific for the type  
25 1 isoform was used for in vivo experiments and had previously been prepared and characterized (Danielpour et al., 1990, Supra). Serum was heat inactivated at 56°C for 30 min. before injection. Animals (5 per group) were injected intraperitoneally (I.P.) with anti-TGF- $\beta$  antiserum or control turkey serum at various concen-  
30 trations (12.5, 25 or 50  $\mu$ l diluted in PBS to a final volume of 100  $\mu$ l), 5 times at days -2, 0, +2, +4, +6 in relationship to MBP/CFA immunization. 1  $\mu$ l of the antiserum blocked 4 mg/ml of binding activity of <sup>125</sup>I-TGF- $\beta$ 1 to A549 cells (Danielpour et al., 1990, Supra). In vivo treatment was given both to orally

tolerized animals and to animals to develop EAE without oral tolerization.

These experiments were performed to examine the effects of anti-TGF- $\beta$  antiserum on oral tolerance induction in vivo, and to see whether TGF- $\beta$  activity was abrogated.

Clinical Evaluation. To examine the correlation between in vitro assays and clinical disease, animals were evaluated in a blinded fashion every day for evidence of EAE. Clinical severity of EAE scored as follows: 0, no disease; 1, limp tail; 2, hind limb paralysis; 3, hind limb paraplegia, incontinence; 4, tetraplegia; 5, death. Duration of disease was measured by counting the total number of days from disease onset (usually days 10 or 11 after immunization) until complete recovery or death for each animal.

Delayed Type Hypersensitivity (DTH) Testing. DTH was tested by injecting 25  $\mu$ g of MBP in PBS subcutaneously in the ear. Thickness was measured before and 48 hours after challenge, by a blinded observer, using micrometer calipers (Mitutoyo, Japan). Change in ear thickness pre-and post-challenge was recorded for each animal and the result expressed as the mean for each experimental group  $\pm$  SEM.

DTH responses were monitored because they are mediated by CD4+ T-cells as is EAE.

Statistical Analysis. Comparisons of means were performed using a one-tailed student t-test and chi square analysis (as is known by those of ordinary skill in the art) was used in comparing the incidence of disease between groups.

Experiments were performed to determine whether supernatants collected from splenocytes depleted of T-cell subsets or B-cells from rats orally tolerized to MBP and stimulated in vitro with MBP could suppress an MBP line. As shown in Figure 1, a reduction in the proliferation of the MBP line occurred with the addition of supernatants from B-cell depleted or CD4 depleted splenocytes from animals fed MBP and stimulated in vitro with MBP. No suppression occurred with

supernatants from cells of Bovine Serum Albumin (BSA) -fed animals or CD8 depleted splenocytes from MBP-fed animals. This indicated that suppression was specific for the fed antigen and required suppressor T-cells.

5           In order to determine whether a known cytokine was responsible for mediating the suppression, neutralizing antibodies to cytokines postulated to have suppressor activity were added to the supernatants in an attempt to abrogate the suppression. As shown in Figure 2, rabbit anti-TGF- $\beta$  antibody abrogated  
10 the suppression mediated by the supernatants in a dose-dependent fashion. No effect on suppression was seen with neutralizing antibodies to INF $\gamma$ , TNF $\alpha$ + $\beta$ , or when indomethacin, a prostaglandin blocker, was added. No suppression occurred when anti-TGF- $\beta$  antibodies were added directly to the MBP specific responder T-  
15 cell line (data not shown). This indicates that TGF- $\beta$  is responsible for the suppression observed in Fig. 1, and was due to a soluble factor.

          In order to directly demonstrate the presence of TGF- $\beta$  in supernatants of spleen cells from animals fed MBP and  
20 stimulated in vitro with MBP, supernatants were collected under serum-free conditions and assayed directly for TGF- $\beta$  as described above. As shown in Figure 3, TGF- $\beta$  was secreted by spleen cells from MBP fed animals stimulated in vitro in the presence, but not in the absence of MBP. Furthermore, TGF- $\beta$  was also secreted when  
25 splenocytes from ovalbumin (OVA) fed animals were stimulated in vitro with OVA. Using a specific SELISA assay with blocking antibodies specific for either TGF- $\beta$ 1 or TGF- $\beta$ 2, it was further demonstrated that TGF- $\beta$  was of the TGF- $\beta$ 1 isotype. In addition, the TGF- $\beta$  secreted was in the active, rather than the latent  
30 form. The amount of TGF- $\beta$  in the group fed and stimulated in vitro with MBP was  $6.8 \pm 1.7$  ng/ml with  $68 \pm 9\%$  in the active form. In the OVA group the amount of TGF- $\beta$  was  $6.1 \pm 1.0$  ng/ml with  $65 \pm 9\%$  in the active form. No active TGF- $\beta$  was observed in supernatants from spleen cells of animals fed MBP and stimulated  
35 with a non-specific inducer of T-cell proliferation,

concanavalin-A (Con-A), although small quantities ( $2.1 \pm 0.45$  ng/ml) of latent TGF- $\beta$  were observed.

In order to determine whether TGF- $\beta$ 1 also played a role in suppression of EAE by oral tolerization to MBP, turkey anti-TGF- $\beta$ 1 anti-serum was administered in vivo. As shown in Figure 4A, paralytic EAE developed in control animals with a maximal disease severity between 3.2-3.5 on day 13 where the animals were injected with PBS or control turkey serum. Oral tolerization with MBP markedly reduced the severity of EAE (Figure 4C) in animals injected with PBS or control turkey serum. Maximal disease severity in animals treated 5 times with 50  $\mu$ l of control serum was  $3.2 \pm 0.2$  and in orally tolerized animals treated 5 times with 50  $\mu$ l of control serum was  $1.0 \pm 0.2$  ( $p < 0.001$ ). As shown in Figure 4D, in vivo treatment with anti-TGF- $\beta$ 1 anti-serum abrogated protection induced by oral administration of MBP in a dose-dependent fashion; maximal disease severity in orally tolerized animals treated 5 times with 50  $\mu$ l of anti-TGF- $\beta$ 1 anti-serum was  $3.7 \pm 0.2$  vs.  $1.0 \pm 0.2$  ( $p < 0.001$ , group D vs. C). Of note is that as shown in Figure 4B, there was a dose-dependent enhancement of disease in animals treated with anti-TGF- $\beta$ 1 anti-serum that were not orally tolerized to MBP. Disease onset was earlier, recovery was delayed, and disease severity was greater ( $4.5 \pm 0.2$  vs.  $3.2 \pm 0.2$ , groups B vs. A  $p < 0.01$ ).

Delayed-type hypersensitivity (DTH) responses correlate with the clinical course of EAE and serve as a measure of in vivo cellular immunity to MBP (Brod, S. A. et al. Ann. Neurol. 29:615-622, 1991; Khoury, S. J. et al. Cell. Immunol. 131:302-310, 1990). DTH responses were tested in the same groups described in Figure 4 by injecting 25  $\mu$ g of MBP in PBS subcutaneously in the ear. Thickness was measured before and 48 hours after challenge. The change in ear thickness pre- and post-challenge was recorded for each animal and the results expressed as the mean for each experimental group  $\pm$  SEM.

As shown in Figure 5 (A-D), prominent DTH responses developed in animals undergoing EAE and DTH responses were

suppressed by oral administration of MBP. The suppressed DTH responses were abrogated by in vivo anti-TGF- $\beta$ 1 treatment in a dose-dependent fashion ( $1.5 \pm 0.5$  vs.  $0.5 \pm 0.3$ ;  $p < 0.001$ , in animals injected 5 times with  $50 \mu\text{l}$  of anti-TGF- $\beta$  vs. control serum). Furthermore, following the same in vivo treatment, there was enhancement of DTH responses to MBP in animals recovering from EAE that were orally tolerized ( $2.1 \pm 0.3$  vs.  $1.4 \pm 0.3$ ;  $p < 0.01$  in animals injected 5 times with  $50 \mu\text{l}$  anti-TGF- $\beta$  vs. control serum).

The results presented above provide evidence for an immunoregulatory role played by endogenous TGF- $\beta$ 1 in the spontaneously occurring recovery from EAE and in the suppression of EAE induced by oral tolerization to MBP. In view of the fact that TGF- $\beta$  features are highly conserved in evolution, it is anticipated that the immunosuppressive effects of TGF- $\beta$  in experimental animals are similar to its effects in humans.

**EXAMPLE 2: Antigen-Driven Bystander Suppression  
After Oral Administration of Antigens**

In the experiments described below, the following materials and methods were used.

Animals. Female Lewis rats 6-8 weeks of age were obtained from Harlan-Sprague Dawley Inc. (Indianapolis, IN). Animals were maintained on standard laboratory chow and water ad libitum.

Antigens. Guinea pig MBP was purified from brain tissue by a method modified from Deibler et al. (supra) as described in Example 1 above and purity was checked by gel electrophoresis. Ovalbumin (OVA) and BSA were purchased from Sigma Chemical Co. (St. Louis, MO) and keyhole limpet hemocyanin (KLH) from Calbiochem Behring Corp. (La Jolla, CA).

Immunization Of Animals. Animals were immunized with  $25 \mu\text{g}$  of MBP in the footpad, emulsified in an equal volume of CFA containing  $4 \text{ mg/ml}$  of Mycobacterium tuberculosis (Difco Labs, Detroit, MI) in order to induce a substantial EAE disease state. For in vivo bystander suppression experiments,  $50\text{-}300 \mu\text{g}$  of the secondary antigens OVA, BSA or KLH were injected subcutaneously

in the same footpad in 100  $\mu$ l PBS 8 hours after primary immunization with MBP CFA.

Clinical Evaluation. Animals were evaluated in a blinded fashion every day for evidence of EAE in order to correlate the clinical manifestations of Bystander Suppression with the in vitro assays described below. Clinical severity of EAE was scored as follows: 0, no disease; 1, limp tail; 2, hind limb paralysis; 3, hind limb paraplegia, incontinence; 4, tetraplegia; 5, death. Mean maximal clinical severity was calculated as previously described for each experimental group (7). Statistical analysis was performed using a one-tailed student's t test and a chi square analysis for comparing incidence between groups.

Induction Of Oral Tolerance. Animals were fed 1 mg MBP, OVA, BSA or KLH dissolved in 1 ml PBS or PBS alone, by gastric intubation using an 18-gauge stainless steel animal feeding needle (Thomas Scientific, Swedesboro, NJ). Animals were fed five times (total dose of 5 mg), at intervals of 2-3 days with the last feeding 2 days before immunization.

Delayed Type Hypersensitivity (DTH) Testing. DTH was tested by injecting 50  $\mu$ g of MBP or OVA in PBS, subcutaneously into the ear. MBP was injected in the left ear and OVA in the right ear in the same animal. Thickness, in units of 0.01 inch, was measured in a blinded fashion, before and 48 hours after challenge, using micrometer calipers (Mitutoyo, Utsunomia, Japan). Change in ear thickness before and after challenge was recorded for each animal, and results were expressed as the mean for each experimental group  $\pm$  SEM; each group consisted of five animals.

Transwell Cultures. A dual chamber transwell culture system (Costar, Cambridge, MA), which is 24.5 mm in diameter and consists of two compartments separated by a semi-permeable polycarbonate membrane, with a pore size of 0.4  $\mu$ m, was used. The two chambers are 1 mm apart, allowing cells to be coincubated in close proximity without direct cell-to-cell contact. To



measure in vitro suppression of proliferative responses in transwell cultures,  $5 \times 10^4$  MBP- or OVA-specific line cells, raised and maintained as previously described (Ben-Nun, A. et al., Eur. J. Immunol. 11:195, 1981), were cultured with  $10^6$  irradiated (2,500 rad) thymocytes, in 600  $\mu$ l of proliferation media in the lower well. Spleen cells from orally tolerized rats or controls (fed BSA) were added to the upper well ( $5 \times 10^5$  cells in 200  $\mu$ l). Spleen cells were removed 7-14 days after the last feeding, and a single cell suspension was prepared by pressing the spleens through a stainless steel mesh. MBP and OVA (50  $\mu$ g/ml) were added in a volume of 20  $\mu$ l. Because modulator cells are separated from responder cells by a semi-permeable membrane, they do not require irradiation. In some experiments, modulator cells were added in the lower well together with responder cells, and in these instances modulator cells were irradiated (1,250 rad) immediately before being placed in culture. Proliferation media consisted of RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with  $2 \times 10^{-5}$  M 2-mercaptoethanol, 1% sodium pyruvate, 1% penicillin and streptomycin, 1% glutamine, 1% HEPES buffer, 1% nonessential amino acids, and 1% autologous serum. Each transwell was performed in quadruplicate. The transwells were incubated at 37°C in a humidified 6% CO<sub>2</sub> and 94% air atmosphere for 72 hours. After 54 hours of culture, each lower well was pulsed with 4  $\mu$ Ci of [<sup>3</sup>H]thymidine and at 72 hours split and reseeded to three wells in a round-bottomed 96-well plate (Costar) for harvesting onto fiberglass filters and counting using standard liquid scintillation techniques. Percent suppression =  $100 \times (1 - \Delta \text{ cpm responders cultured with modulators} / \Delta \text{ cpm of responders})$ .

The transwell system was used to examine the soluble factors produced during Bystander Suppression and to monitor the transfer of suppression during the process.

Purification Of T-Cell Subsets. Depletion of T-cell subsets was performed by negative selection using magnetic beads according to the modified method of Cruikshank et al., supra.

Spleen cells were incubated with a 1:100 dilution of mouse anti-rat CD8, or CD4, mAbs (clones OX/8 or W3/25 Serotec/Bioproducts, Indianapolis, IN) for 30 minutes on ice, washed twice, and then added to prewashed magnetic particles, with an average diameter of 450 microns (M-450) with goat anti-mouse IgG covalently attached (Dynal Inc., Fort Lee, NJ). The quantity of magnetic beads used was calculated as being 10 times the estimated target cell population. The cells were incubated with the beads in 0.5 ml of RPMI 1640 supplemented with 10% FCS in a 10 ml round-bottomed test tube (Nunc, Roskilde, Denmark) for 30 minutes on ice with gentle shaking every 5 minutes. After incubation, the bead/cell suspension was washed with 5 ml of medium and cell-mAb-bead complexes were separated from unlabeled cells in a strong magnetic field using a magnetic-particle concentrator (Dynal-MPC-1) for 2 minutes. The supernatant was removed, and the procedure repeated twice to obtain the nonadherent fraction. The T-cells in the depleted population were 95% CD4<sup>+</sup>CD8<sup>-</sup> or CD8<sup>+</sup>CD4<sup>-</sup> as demonstrated by indirect flow cytometry.

Adoptive Transfer Of Disease Suppression. In order to monitor the adoptive transfer of disease suppression occurring during Bystander Suppression donor rats were fed either 1 mg MBP, OVA, or KLH, five times at 2 day intervals and killed 7-14 days after the final feeding. Spleen cells were harvested, and incubated in vitro with the homologous antigen (50 µg/ml) in proliferation medium, for 72 hours. Cells were injected intraperitoneally: 10<sup>8</sup> cells for whole spleen populations or 5-6 x 10<sup>7</sup> cells for CD8<sup>-</sup> or CD4<sup>-</sup> depleted populations. Recipient animals were irradiated (250 rad) before adoptive transfer, immunized with MBP/CFA 6 hours after adoptive transfer, and challenged 8 hours later with 50 µg OVA.

To determine whether cell-to-cell contact was required for in vitro suppression to occur, a transwell system (described above) was used. The results are set forth in Table 2 below.

As shown in Table 2, when irradiated splenocytes from MBP-fed animals were incubated together with an MBP line in the

lower well, there was suppression of proliferation (line 2), while no suppression was observed with splenocytes from PBS fed animals (line 3). Virtually identical suppression was observed when modulator cells were separated from responder cells by the  
5 semipermeable membrane (lines 4 and 5). Thus, suppression appeared to be mediated by a soluble factor or factors that diffuse through the transwell membrane. Therefore, Bystander Suppression appeared to be operative in the induction of oral tolerance in EAE.

Table 2. Suppression of an MBP T Cell Line by Spleen Cells from MBP-fed Donors in Transwell System

5	Upper well	Lower well	$\Delta$ cpm	Percent Suppression
10	1. -	MBP line	37,809 $\pm$ 3,326	
	2. -	MBP line + MBP-fed modulators	18,412 $\pm$ 1,867	51
	3. -	MBP line + PBS-fed modulators	34,631 $\pm$ 3,994	8
15	4. MBP-fed modulators	MBP line	15,620 $\pm$ 2,294	59
	5. PBS-fed modulators	MBP line	34,043 $\pm$ 3,731	10

20  $5 \times 10^4$  MBP line cells + MBP (50  $\mu\text{g/ml}$ ) were placed in the lower well with  $10^6$  irradiated (2,500 rad) thymocytes as antigen presenting cells (APC). Splenic modulator cells ( $5 \times 10^5$ ) from MBP- or PBS-fed animals were added to either the upper or lower well. Modulator cells added to the lower well were irradiated (1,250 rad). Background counts of the MBP line without MBP added were between 1,000 and 2,000 cpm.

To determine whether that in vitro suppression observed in the transwell system required identical antigen specificity between modulator and responder cells, an OVA line was placed in the lower well. The results are set forth in Table 3 below.

- 5           As shown in Table 3, modulator cells from MBP-fed animals placed in the upper well were able to suppress an OVA line in the lower well, in the presence, but not in the absence, of MBP (lines 2 and 3). MBP added to modulator cells from animals fed PBS did not suppress the OVA line (line 4).
- 10   Conversely, suppression of an MBP line was seen with modulator cells from OVA-fed animals in the presence of OVA (line 7). Of note is that soluble antigen added to the transwell in either well diffused across the membrane and thus was present in both wells as would be the case in vivo.

Table 3. Suppression of an OVA or MBP T-Cell Line by Spleen Cells from MBP- or OVA-fed Donors in Transwell System

	5	Modulator (upper well)	Responder (lower well)	$\Delta$ cpm	Percent Suppression
	10	1. -	OVA line + OVA	62,761 $\pm$ 3,881	-
		2. MBP-fed	OVA line + OVA	65,868 $\pm$ 3,989	-5
		3. MBP-fed + MBP	OVA line + OVA	30,974 $\pm$ 3,450	51
	15	4. PBS-fed + MBP	OVA line + OVA	61,132 $\pm$ 2,967	<1
		5. -	MBP line + MBP	71,503 $\pm$ 4,581	-
		6. OVA-fed	MBP line + MBP	67,075 $\pm$ 2,904	6
	20	7. OVA-fed + OVA	MBP line + MBP	37,778 $\pm$ 3,780	47
		8. PBS-fed + OVA	MBP line + MBP	68,104 $\pm$ 4,832	5

25 5 x 10<sup>4</sup> MBP or OVA line cells were placed in the lower well with 10<sup>6</sup> irradiated (2,500 rad) thymocyte as APC. Modulator cells (5 x 10<sup>5</sup>) from MBP-, OVA- or PBS-fed animals were added to the upper well. Background counts of the MBP and OVA lines without MBP or  
30 OVA added were between 1,000 and 2,000 cpm.

To determine the relationship between the above in vitro bystander suppression and the in vivo situation, a series of experiments were conducted in the EAE model. Rats were fed OVA (1 mg, five times over a 10 day period), then immunized with  
35 MBP/CFA in the footpad and given OVA 8 hours later in the same footpad. As shown in Figure 6A, injecting OVA in the footpad 8 hours after immunization with MBP/CFA had no effect on EAE as expected. Mean maximal clinical disease severity was 3.9  $\pm$  0.2 for MBP/CFA immunized and 3.8  $\pm$  0.1 with OVA given subcutane-  
40 ously. However, in animals fed OVA before immunization with MBP/CFA after which OVA was given subcutaneously in the footpad, suppression of EAE occurred in an analogous fashion to feeding MBP (Figure 6B); disease severity in OVA fed plus OVA given subcutaneously was 0.9  $\pm$  0.2, in MBP fed it was 1.1  $\pm$  0.1, and in

the OVA fed and KLH given subcutaneously (control group)  $3.9 \pm 0.1$  ( $p < 0.001$ , OVA and MBP fed vs. control). Therefore, CD4+ T-cells induced by immunization with MBP/CFA were down regulated by TGF- $\beta$  released by CD8+ T-cells induced by oral administration of a bystander antigen, in this case OVA. No suppression of EAE was observed in animals fed OVA in whom KLH was given after MBP/CFA plus OVA subcutaneously (Figure 6C), disease severity was  $3.7 \pm 0.1$  and  $3.8 \pm 0.2$ , respectively. These experiments demonstrate an in vivo effect similar to that seen in vitro in the transwell system. Specifically, modulator cells generated by oral tolerization to one antigen can suppress cells of a different antigen specificity when the tolerizing antigen is present.

To determine whether a correlation existed in the in vivo bystander system and to determine the degree of sensitization that occurs in association with the bystander effect, DTH responses were measured. Suppressed DTH responses to MBP were observed both in animals fed MBP and those fed OVA that were subsequently immunized with the MBP/CFA plus OVA (Figure 7). Oral administration of other antigens, such as KLH or BSA, had no effect on DTH responses to MBP in these animals. Feeding OVA followed by the injection of OVA subcutaneously in association with MBP/CFA did not generate an immune response to OVA as measured by DTH.

To rule out the possibility that something unique to OVA was responsible for the in vivo bystander suppression observed, similar experiments were conducted in which BSA was fed and then given subcutaneously after MBP/CFA immunization. As shown in Figure 8, oral administration of BSA prior to immunization with MBP/CFA followed by BSA (the bystander antigen) given subcutaneously suppressed EAE in an analogous fashion as that seen with OVA. Of note is that suppression of EAE associated with BSA was observed only when the secondary antigen was given subcutaneously at a dose of 300  $\mu$ g, whereas with OVA, suppression occurred at a dose of 50  $\mu$ g.

As shown in Figure 9, spleen cells from MBP- or OVA-fed animals adoptively transferred protection into naive recipients, which were immunized with MBP/CFA and given OVA subcutaneously. Furthermore, adoptively transferred suppression was abrogated by depletion of CD8<sup>+</sup> (suppressor T-cells), but not by depletion of CD4<sup>+</sup> cells. No protection was observed with the adoptive transfer of spleen cells from KLH-fed animals to animals immunized with MBP/CFA plus OVA.

10 **EXAMPLE 3: Identification of Immunosuppressive  
Epitopes of Guinea Pig MBP**

The Transwell System of Example 2 above was used to identify the epitopes present on guinea pig MBP which induce the release of TGF- $\beta$  from suppressor T-cells.

The disease-inducing fragments (autoimmune response epitopes) of MBP were first confirmed as follows: Overlapping peptides as detailed in Figure 10, of guinea pig MBP were obtained from commercial sources or synthesized in accordance with well-known techniques, specifically using a commercial peptide synthesis apparatus (from Applied Biosystems) and following the manufacturer's instructions. Whole MBP was then fed to rats and lymph node cells from the orally tolerized animals were triggered with the MBP-peptides. The ability of the triggered cells to induce killer T-cells was then quantitatively determined by a proliferation assay also, as described in Examples 1 and 2, and by testing the ability of the proliferating cells to transfer the disease.

As shown in Figure 10, a peptide spanning residue 71-90 of guinea pig MBP was by far the most efficient inducer of killer T-cells and therefore the most potent disease-promoting fragment of MBP. This region of guinea pig MBP therefore corresponds to the immunodominant epitope of the protein.

When spleen cells obtained from animals fed MBP and immunized with MBP/CFA (as described above in Examples 1 and 2) were co-cultured in the transwell system with spleen cells isolated from OVA-fed animals, peptides corresponding to guinea pig MBP amino acid residues 21-40, 51-70 and 101-120 added to the



modulator well were all capable of triggering suppression of proliferation of the OVA-fed line. The results are shown in Figure 11. Of note is the fact that the immunodominant epitope of guinea pig MBP, identified in Figure 10 and 11 (corresponding to amino acid residue nos. 71-90) was ineffective in triggering suppression in the transwell system. Peptides corresponding to guinea pig MBP residue nos. 151-170 and 161-178 inhibited proliferation of the OVA (responder) line but this effect was non-specific, and may have been due to toxicity induced in vitro by these peptides, as these same peptides inhibited proliferation of spleen cells isolated OVA-fed animals when co-cultured with control (non-MBP-fed) modulator cells (data not shown). These experiments further demonstrated that feeding antigens which elicit TGF- $\beta$  to the animals is required for bystander suppression. These experiments also demonstrated that the portions of an autoantigen that are involved in bystander suppression are different from those involved in autoimmune response.

**EXAMPLE 4: Cells, Cytokines and Activation Markers  
in Sections of Rat Brain Obtained from  
Normal EAE-induced and MBP-fed Rats**

The effect of oral administration of MBP in rats induced for EAE was analyzed in terms of the cells and factors present in the brains of fed and control rats. Lewis rats were fed MBP five times then immunized with MBP/CFA and their brains were examined immunohistologically at day 14 (peak of disease) and compared to brains from immunized control-fed animals harvested at the same time, and to brains of naive animals. Cryostat sections of cortex and cerebellum were fixed in paraformaldehyde-lysine-periodate for determination of leukocytes and activation antigens, or in acetone for the labelling of cytokines, and stained by a peroxidase-antiperoxidase method (Hancock, W.W., et al., J. Immunol. 138:164, 1987). Results of cytokine and endothelial labelling in 20 consecutive fields were judged as (-) absence of labelling, (+/-) <10 cells/section or trace labelling, (+) few small foci, (2+) multiple foci, and (3+)

multiple large perivascular collections and diffuse submeningeal staining.

The results are summarized in Table 4.

**TABLE 4**

5                      **Cells, Cytokines and Activation Markers  
in Sections of Rat Brain (n=3/group)**

10	<u>Marker</u>	<u>Normal</u>	<u>EAE- Induced Rats</u>	<u>MBP- Fed Rats</u>	<u>MBP/LPS- Fed Rats</u>
	Leukocytes, CD4+MNC	+/-	3+	1+	1+
	IL-2R(p55), PCNA	-	2+	+/-	-
	IL-1, IL-2, IL-6 IFN- $\gamma$ , TNF	+/-	2+	1+	-
15	IL-4	-	-	-	2+
	TGF- $\beta$	-	-	2+	2+
	PGE	-	+/-	-	2+
	Ia, ICAM-1	+/-	3+	1+	1+

20                      In the MBP-fed group there was evidence of  
downregulation of cellular inflammatory immune response and TNF,  
Ia and ICAM-1 expression, while there was upregulation of TGF- $\beta$   
expression (Table 4).

25                      It has been discovered previously that  
lipopolysaccharide (LPS) enhances suppression of EAE achieved by  
oral administration of MBP. Thus, the brains of MBP+LPS fed  
animals at the peak of the disease was also examined and in  
addition to the changes observed with MBP feeding alone there was  
no upregulation of IL-4 and PGE expression. Therefore, under  
certain conditions IL-4 or other regulatory cytokines participate  
30 with TGF- $\beta$  in down-regulation of the immune response.

Feeding synergist alone (without bystander or  
autoantigen) does not result in upregulation of IL-4 or PGE (data  
not shown).

35                      In summary, as can be seen from the results set forth  
in Table 4 above, normal rat brains do not contain cells,  
cytokines and activation markers, whereas EAE-induced rats have  
various inflammatory cells and inflammatory cytokines (i.e. IL-1,

IL-2, IL-6, IFN- $\gamma$  and TNF) present. In contrast, EAE-induced rats which were fed MBP plus LPS (a synergist) have a reduction of the cells and inflammatory cytokines and, in addition, contained suppressor T-cells (CD8+ subset), IL-4, TGF- $\beta$  and  
5 prostaglandin E(PGE), all of which counter the actions of the CD4+MNC and inflammatory cytokines.

**EXAMPLE 5:**      **Suppression of Insulitis in NOD Mice By Oral Administration of Insulin Peptides and Glucagon**

The effect of feeding separated insulin A- or B-chain  
10 and various synthetic peptides derived from the insulin B-chain protein molecule and of glucagon on insulitis in NOD mice was studied.

NOD mice (Taconic Labs) were fed one mg of glucagon, or one mg of porcine insulin (both commercially purchased) or equal  
15 molar amounts of insulin A-chain, B-chain and B-chain peptides described below (all insulin fragments having been synthesized) twice weekly for five weeks and sacrificed at ten weeks of age.

Control animals were fed a non-pancreatic (i.e.,  
20 unrelated) peptide, GAP. Insulitis was measured as a semiquantitative insulitis score according to the method described in Zhang, Z. J., PNAS(USA), 88:10252-10256, 1991.

The insulin B-chain peptides corresponded to amino acid residues 1-12 (B<sub>1-12</sub>), 10-22 (B<sub>10-22</sub>), 11-30 (B<sub>11-30</sub>) and 23-30 (B<sub>23-30</sub>).  
25 All animals were fed 10 times over three weeks.

The results are set forth in Table 5 below.

TABLE 5

	<u>Group</u>	<u>Amount (mg)</u>	<u>Insulinitis Score</u>
	Control (PBS-Fed)		2.66
	A-Chain-Fed	0.4	1.88
5	B-Chain-Fed	0.6	1.32
	B <sub>1-12</sub> -Fed	0.24	1.76
	B <sub>10-22</sub> -Fed	0.27	1.71
	B <sub>11-30</sub> -Fed	0.40	1.44
	B <sub>23-30</sub> -Fed	0.17	2.22
10	MBP-Fed	1	2.14
	GAP-Fed	0.24	2.47
	Glucagon-Fed	1	1.81

As can be seen from the results set forth Table 6 above, the insulin A-chain or B-chain suppressed insulinitis, with B-chain feeding showing a greater degree of suppression. Peptides B<sub>1-12</sub>, B<sub>10-22</sub> and B<sub>11-30</sub> also suppressed insulinitis whereas B<sub>23-30</sub> did not. No suppression was observed in animals fed with MBP or GAP. In addition, glucagon, a Bystander antigen, was also effective in suppressing insulinitis.

20 EXAMPLE 6: Oral Tolerance vs. IV Administration of Bovine-PLP or Mouse MBP

In order to compare the effect of tolerization via the oral or the intravenous (IV) route of administration and to further demonstrate bystander suppression, groups of 5-6 female, 7 week old, SJL/J mice (Jackson Labs, Bar Harbor, ME) were immunized with PLP peptide 140-160 on days 0 and 7 and received the following treatments:

GROUPS

1. Fed Histone (0.25 mg)
2. Fed Mouse MBP (0.25 mg)
3. Fed Bovine PLP (0.25 mg)
4. Inject I.V. Histone (0.25 mg)
5. Inject I.V. MBP (0.25 mg)
6. Inject I.V. PLP (0.25 mg)

Each group was treated every other day for 7 days. In the intravenous group, the material was injected into the eye

plexus. The PLP peptide used was the disease inducing fragment 140-160 of bovine PLP. This peptide has the amino acid sequence COOH-PLAYTIGVFKDPHGLWKGLCNH<sub>2</sub>, representing the foregoing amino acid residues.

5 As shown in Figure 12, both mouse MBP and bovine PLP were equally effective in down-regulating PLP-peptide-induced EAE when orally administered. A non-specific protein, histone, was ineffective in suppressing EAE when administered orally. Thus, a bystander antigen, in this case mouse MBP, effectively  
10 suppressed EAE when orally administered to animals induced for EAE with bovine PLP.

In contrast, when administered intravenously, only the antigen used to induce the disease, in this case bovine PLP, was effective in suppressing EAE. The results are shown in Figure  
15 13.

The effects of feeding various peptides to Lewis rats induced for EAE by guinea pig MBP residue nos. 71-90 (the major immunodominant epitope of guinea pig MBP as shown in Example 3 above) were also studied.

20 EAE was induced by immunizing with 0.25 mg of guinea pig MBP amino acid residue nos 71-90 in Complete Freund's Adjuvant and the effect of feeding various guinea pig MBP peptides on EAE was examined.

As shown in Fig. 14, whole guinea pig MBP and a 21-40  
25 guinea pig peptide were equally effective in downregulating EAE induced by guinea pig MBP 71-90 as was 71-90. Guinea pig MBP peptide 131-150 was ineffective in this case. Peptides were also fed with STI which prevents their breakdown by gastric juices and enhances their biological effect. DTH responses to whole MBP  
30 were suppressed by feeding MBP or any one of the MBP-peptides 21-40, or 71-90. However, DTH responses to guinea pig MBP peptide 71-90 were only suppressed by feeding either whole MBP or guinea pig peptide 71-90 and were not affected by guinea pig MBP peptide 21-40 (Fig. 15). This is consistent with the conclusion that MBP  
35 fragment 71-90 does not participate in bystander suppression.

Finally, the suppression of EAE by I.V. tolerization with MBP and MBP peptides prior to disease expression (on days 8 and 9 post immunization) was examined.

As shown in Figure 16, in animals induced for EAE with whole guinea pig MBP, only whole guinea pig MBP and guinea pig MBP peptides corresponding to amino acid residues 71-90 were effective in suppressing EAE when administered via the I.V. route. Peptides corresponding to guinea pig MBP amino acid residue nos. 21-40, which are effective in downregulating EAE when administered orally, were ineffective in suppressing EAE when administered intravenously, consistent with the inability of IV administration to trigger bystander suppression. A peptide corresponding to amino acid residue nos. 131-150 and histone were also ineffective in suppressing EAE when administered intravenously, consistent with the fact that neither of these antigens is responsible for autoimmune response

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APPENDIX A

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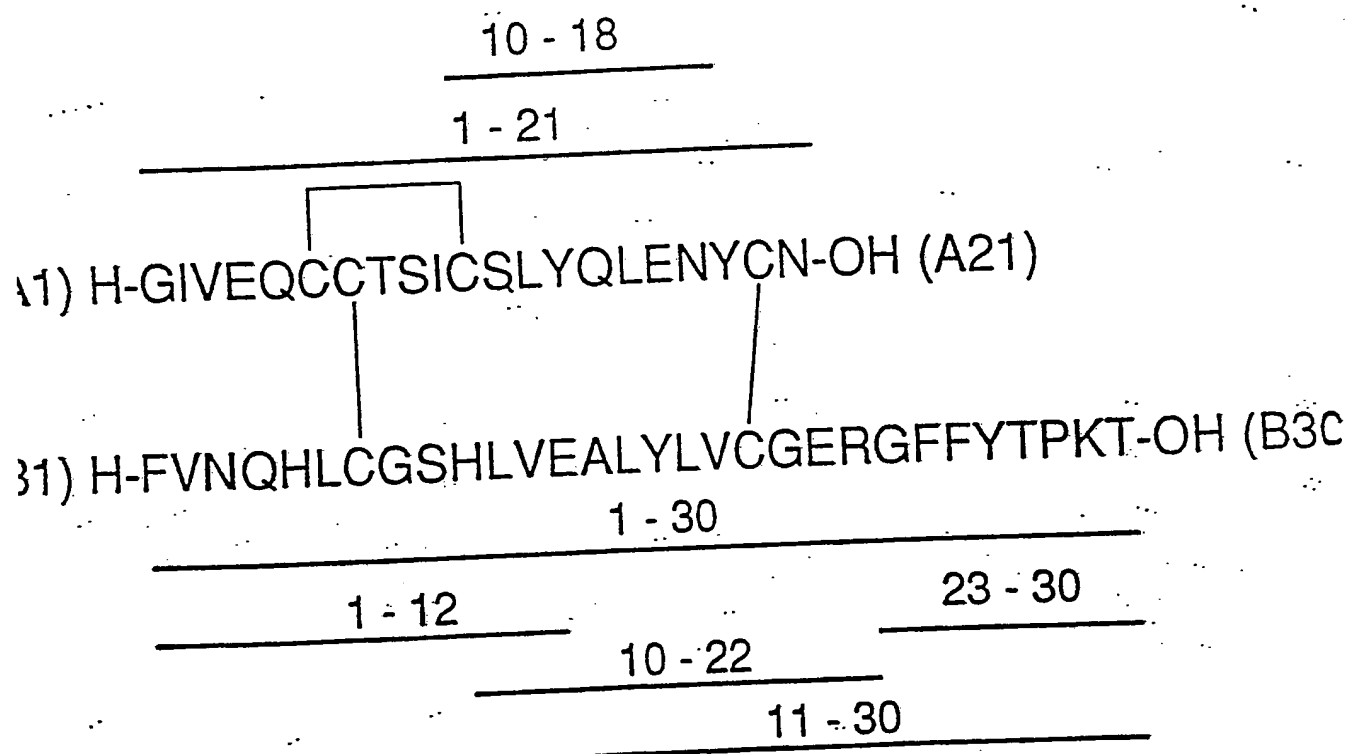
10          20
Gly-Leu-Leu-Glu-Cys-Cys-Ala-Arg-Cys-Leu-Val-Gly-Ala-Pro-Phe-Ala-Ser-Leu-Val-Ala-
.....
30          40
Thr-Gly-Leu-Cys-Phe-Phe-Gly-Val-Ala-Leu-Phe-Cys-Gly-Cys-Gly-His-Glu-Ala-Leu-Thr-
.....
50          60
Gly-Thr-Glu-Lys-Leu-Ile-Glu-Thr-Tyr-Phe-Ser-Lys-Asn-Tyr-Glu-Asp-Tyr-Glu-Tyr-Leu-
51.....
70          80
Ile-Asn-Val-Ile-His-Ala-Phe-Glu-Tyr-Val-Ile-Tyr-Gly-Thr-Ala-Ser-Phe-Phe-Phe-Leu-
.....
90          100
Tyr-Gly-Ala-Leu-Leu-Leu-Ala-Tyr-Gly-Phe-Tyr-Thr-Thr-Gly-Ala-Val-Arg-Glu-Ile-Phe-
.....
110         120
Gly-Asp-Tyr-Lys-Thr-Thr-Ile-Cys-Gly-Lys-Gly-Leu-Ser-Ala-Thr-Val-Thr-Gly-Gly-Gln-
52.....
130         140
Lys-Gly-Arg-Gly-Ser-Arg-Gly-Gln-His-Glu-Ala-His-Ser-Leu-Glu-Arg-Val-Cys-His-Cys-
.....
150         160
Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys-Phe-Val-Gly-Ile-Thr-Tyr-Ala-Leu-Thr-Val-
.....
170         180
Val-Trp-Leu-Leu-Val-Phe-Ala-Cys-Ser-Ala-Val-Pro-Val-Tyr-Ile-Tyr-Phe-Asn-Thr-Trp-
.....
190         200
Thr-Thr-Cys-Glu-Ser-Ile-Ala-Ala-Pro-Ser-Lys-Thr-Ser-Ala-Ser-Ile-Gly-Thr-Leu-Cys-
Phe  x Ser
.....
210         220
Ala-Asp-Ala-Arg-Met-Tyr-Gly-Val-Leu-Pro-Trp-Asn-Ala-Phe-Pro-Gly-Lys-Val-Cys-Gly-
.....
230         240
Ser-Asn-Leu-Leu-Ser-Ile-Cys-Lys-Thr-Ala-Glu-Phe-Glu-Met-Thr-Phe-His-Leu-Phe-Ile-
Val Asn
.....
250         260
Ala-Ala-Phe-Val-Gly-Ala-Ala-Ala-Thr-Leu-Val-Ser-Leu-Val-Thr-Phe-Met-Ile-Ala-Ala-
.....
270
Thr-Tyr-Asn-Phe-Ala-Val-Leu-Lys-Leu-Met-Gly-Arg-Gly-Thr-Lys-Phe
.....

```

Sequence of bovine PLP.



## INSULIN FRAGMENTS



		1		2		3																													
		0		0		0																													
Hum	A	S	C	K	R	P	S	Q	R	N	C	S	K	T	L	A	T	A	S	T	H	D	H	A	R	H	G	F	L	P	R	R	H	D	T
Bov	A	A	O	K	R	P	S	Q	R	N	C	S	K	T	L	A	T	A	S	T	H	D	H	A	R	H	G	F	L	P	R	R	H	D	T
Rab	A	S	C	K	R	P	S	Q	R	N	C	S	K	T	L	A	T	A	S	T	H	D	H	A	R	H	G	F	L	P	R	R	H	D	T
GPig	A	S	C	K	R	P	S	Q	R	N	C	S	K	T	L	A	T	A	S	T	H	D	H	A	R	H	G	F	L	P	R	R	H	D	T
RatS	A	S	C	K	R	P	S	Q	R	N	C	S	K	T	L	A	T	A	S	T	H	D	H	A	R	H	G	F	L	P	R	R	H	D	T
Chic	A	S	C	K	R	P	S	Q	R	N	C	S	K	T	L	A	T	A	S	T	H	D	H	A	R	H	G	F	L	P	R	R	H	D	T

		4		5		6		7																											
		0		0		0		0																											
Hum	G	I	L	D	S	I	C	R	F	F	G	C	D	R	C	A	P	E	R	R	S	S	K	D	S	H	E	P	A	R	T	A	N	V	C
Bov	S	I	L	D	S	I	C	R	F	F	G	C	D	R	C	A	P	E	R	R	S	S	K	D	S	H	E	P	A	R	T	A	N	V	C
Rab	G	I	L	D	S	I	C	R	F	F	G	C	D	R	C	A	P	E	R	R	S	S	K	D	S	H	E	P	A	R	T	A	N	V	C
GPig	G	I	L	D	S	I	C	R	F	F	G	C	D	R	C	A	P	E	R	R	S	S	K	D	S	H	E	P	A	R	T	A	N	V	C
RatS	G	I	L	D	S	I	C	R	F	F	G	C	D	R	C	A	P	E	R	R	S	S	K	D	S	H	E	P	A	R	T	A	N	V	C
Chic	G	I	L	D	S	I	C	R	F	F	G	C	D	R	C	A	P	E	R	R	S	S	K	D	S	H	E	P	A	R	T	A	N	V	C

		8		9		10																													
		0		0		0																													
Hum	S	L	P	Q	K	S	-	H	E	R	T	C	D	R	E	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	P	S	Q	C
Bov	S	L	P	Q	K	S	-	H	E	R	T	C	D	R	E	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	P	S	Q	C
Rab	S	L	P	Q	K	S	-	H	E	R	T	C	D	R	E	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	P	S	Q	C
GPig	S	L	P	Q	K	S	-	H	E	R	T	C	D	R	E	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	P	S	Q	C
RatS	S	L	P	Q	K	S	-	H	E	R	T	C	D	R	E	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	P	S	Q	C
Chic	S	L	P	Q	K	S	-	H	E	R	T	C	D	R	E	P	V	V	H	F	F	K	N	I	V	T	P	R	T	P	P	P	S	Q	C

		11		12		13		14																									
		0		0		0		0																									
Hum	K	E	R	G	L	S	L	S	R	F	S	W	G	A	Z	C	O	X	F	G	F	G	C	R	A	S	D	Y	K	S	A	N	K
Bov	K	E	R	G	L	S	L	S	R	F	S	W	G	A	Z	C	O	X	F	G	F	G	C	R	A	S	D	Y	K	S	A	N	K
Rab	K	E	R	G	L	S	L	S	R	F	S	W	G	A	Z	C	O	X	F	G	F	G	C	R	A	S	D	Y	K	S	A	N	K
GPig	K	E	R	G	L	S	L	S	R	F	S	W	G	A	Z	C	O	X	F	G	F	G	C	R	A	S	D	Y	K	S	A	N	K
RatS	K	E	R	G	L	S	L	S	R	F	S	W	G	A	Z	C	O	X	F	G	F	G	C	R	A	S	D	Y	K	S	A	N	K
Chic	K	E	R	G	L	S	L	S	R	F	S	W	G	A	Z	C	O	X	F	G	F	G	C	R	A	S	D	Y	K	S	A	N	K

		15		16		17																													
		0		0		0																													
Hum	G	F	X	G	-	V	-	D	A	O	G	T	L	S	K	I	F	K	L	G	R	-	-	-	D	S	R	S	G	S	P	H	A	R	R
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GPig	G	F	X	G	-	A	-	D	A	O	G	T	L	S	K	I	F	K	L	G	R	-	-	-	D	S	R	S	G	S	P	H	A	R	R
RatS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
Chic	G	N	X	G	T	S	R	Q	(G,E,G)	T	L	S	K	I	F	K	L	G	R	F	(S,G,S,G,S)	R	S	G	S	P	H	A	R	R					

Amino acid sequences of myelin basic proteins derived from human (Hum), bovine (Bov), rabbit (Rab), guinea pig (GPig), rat (RatS), and chicken (Chic) central nervous system tissue. The rat BP used was the 14 kDa BP (rat small, or RatS) which has a deletion of residues 118-159, coded for by the 6th exon of the BP gene. Both the human 18.5 and 17.2 kDa forms of BP were used. The latter has a deletion of residues 107-117 (underlined), coded for by the 5th exon of the BP gene. The sequences are arranged such that homologous residues from each species are arranged vertically, so that they can easily be compared with one another. This system accommodates the deletions and additions that are found among the species, and allows for a total of 177 potential sites among the different molecules. The sequences of residues in parentheses have not been established.

## The comparative amino acid sequences:

Human $\alpha 1$ (II)	Bovine $\alpha 1$ (II)	Bovine $\alpha 1$ (I)	
gly pro met	gly VAL met	gly pro met	105
gly pro met	gly pro met	gly pro SER	
gly pro arg	gly pro arg	gly pro arg	
gly pro pro	gly pro pro*	gly LEU pro*	
gly pro ala	gly pro ala	gly pro PRO*	
gly ala pro	gly ala pro*	gly ala pro*	
gly pro gln	gly pro gln	gly pro gln	
gly phe gln	gly phe gln	gly phe gln	
gly asn pro	gly asn pro	gly PRO pro	
gly glu pro	gly glu pro*	gly glu pro*	132
gly glu pro	gly glu pro*	gly ala pro*	
gly val ser	gly val ser	gly ALA ser	
gly pro met	gly pro met	gly pro met	
gly pro arg	gly pro arg	gly pro arg	
gly pro pro	gly pro pro*	gly pro pro*	
gly pro pro	gly pro pro*	gly pro pro*	
gly lys pro	gly lys pro*	gly lys ASN	
gly asp asp	gly asp asp	gly asp asp	
gly glu ala	gly glu ala	gly glu ala	
gly lys pro	gly lys pro*	gly lys pro*	162
gly lys ala	gly lys SER	gly ARG PRO*	
gly glu arg	gly glu arg	gly glu arg	
gly pro pro	gly pro pro*	gly pro pro*	
gly pro gln	gly pro gln	gly pro gln	
gly ala arg	gly ala arg	gly ala arg	
gly phe pro	gly phe pro*	gly LEU pro*	
gly thr pro	gly thr pro*	gly thr ALA	
gly leu pro	gly leu pro*	gly leu pro*	
gly val lys	gly val lys*-glc-gla	gly MET lys*-glc-gla	
gly his arg	gly his arg	gly his arg	192
gly tyr pro	gly tyr pro*	gly PHE SER	
gly leu asp	gly leu asp	gly leu asp	
gly ala lys	gly ala lys*-glc-gla	gly ala lys*-glc-gla	
gly glu ala	gly glu ala	gly ASP ala	
gly ala pro	gly ala pro*	gly PRO ALA	
gly val lys	gly val lys	gly PRO lys	
gly glu ser	gly glu ser	gly glu PRO*	
gly ser pro	gly ser pro*	gly ser pro*	
gly glu asn	gly glu asn	gly glu asn	
gly ser pro	gly ser pro*	gly ALA pro*	222
gly pro met	gly pro met	gly GLN met	
gly pro arg	gly pro arg	gly pro arg	
gly leu pro	gly leu pro*	gly leu pro*	
gly glu arg	gly glu arg	gly glu arg	
gly arg thr	gly arg thr	gly arg PRO*	

gly pro ala	gly pro ala	gly pro PRO*	
gly ala ala	gly ala ala	gly SER ala	
gly ala arg	gly ala arg	gly ala arg	
gly asn asp	gly asn asp	gly ASP asp	
gly gln pro	gly gln pro*	gly ALA VAL	252
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gly pro pro	gly pro pro*	gly pro pro*	
gly pro val	gly pro val	gly pro THR	
gly pro ala	gly pro ala	gly pro ala	
gly gly pro	gly gly pro*	gly PRO pro*	
gly phe pro	gly phe pro*	gly phe pro*	
gly ala pro	gly ala pro*	gly ala VAL	
gly ala lys	gly ala lys*-glc-gla	gly ala lys*-glc-gla	
gly glu ala	gly glu ala	gly glu GLY	
gly pro thr	gly pro thr	gly pro thr	282
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gly pro glu	gly pro glu	gly SER glu	
gly ala gln	gly ala gln	gly PRO gln	
gly pro arg	gly pro arg	gly VAL arg	
gly glu pro	gly glu pro*	gly glu pro*	
gly thr pro	gly thr pro*	gly PRO pro*	
gly ser pro	gly ALA pro*	gly PRO ALA	
gly pro ala	gly pro ala	gly ALA ala	
gly ala ser	gly ala ALA	gly PRO ALA	
gly asn pro	gly asn pro*	gly asn pro*	312
gly thr asp	gly ALA asp	gly ALA asp	
gly ile pro	gly ile pro*	gly GLU pro*	
gly ala lys	gly ala lys*	gly ala lys*	
gly ser ala	gly ser ala	gly ALA ASN	
gly ala pro	gly ala pro*	gly ala pro*	
gly ile ala	gly ile ala	gly ile ala	
gly ala pro	gly ala pro*	gly ala pro*	
gly phe pro	gly phe pro*	gly phe pro*	
gly pro arg	gly ALA arg	gly ALA arg	
gly pro pro	gly pro pro*	gly pro SER	342
asp pro gln	GLY PRO THR	GLY PRO GLN	
gly ala thr	gly ala SER	gly ala PRO	
gly pro leu	gly pro leu	gly pro PRO*	
gly pro lys	gly pro lys*	gly pro lys*	
gly gln thr	gly gln thr	gly ASN SER	
gly lys pro	gly lys pro	gly lys pro	
gly ile ala	gly ile ala	gly ALA PRO*	
gly phe lys	gly phe lys*	gly ASN lys*	
gly glu gln	gly glu gln	gly ASP THR	
gly pro lys	gly pro lys*	gly ALA lys*	372
gly glu pro	gly glu pro*	gly glu pro*	
gly pro ala	gly pro ala	gly pro THR	
gly pro gln	gly VAL gln	gly ILE gln	
gly ala pro	gly ala pro*	gly PRO pro*	

gly pro ala  
gly glu glu  
gly lys arg  
gly ala arg  
gly glu pro  
gly gly val  
gly pro ile  
gly pro pro  
gly glu arg  
gly ala pro  
gly asn arg  
gly phe pro  
gly gln asp  
gly leu ala  
gly pro lys  
gly ala pro  
gly glu arg  
gly pro ser  
gly leu ala  
gly pro lys  
gly ala asn  
gly asp pro  
gly arg pro  
gly glu pro  
gly leu pro  
gly ala arg  
gly leu thr  
gly arg pro  
gly asp ala  
gly pro gln  
gly lys val  
gly pro ser  
gly ala pro  
gly glu asp  
gly arg pro  
gly pro pro  
gly pro gln  
gly ala arg  
gly gln pro  
gly val met  
gly phe pro  
gly pro lys  
gly ala asn  
gly glu pro  
gly lys ala  
gly glu lys  
gly leu pro  
gly ala pro  
gly leu arg

gly pro ala  
gly glu glu  
gly lys arg  
gly ala arg  
gly glu pro\*

gly gly ALA  
gly pro ALA

gly pro pro\*  
gly glu arg  
gly ala pro\*

gly SER arg

gly phe pro\*

gly gln asp

gly leu ala

gly pro lys\*

gly PRO pro\*

gly glu arg

gly SER PRO\*

gly ALA VAL

gly pro lys\*

gly SER PRO\*

gly GLU ALA

gly arg pro\*

gly glu ALA

gly leu pro\*

gly ala LYS\*

gly leu thr

gly arg pro\*

gly asp ala

gly pro gln

gly lys val

gly pro ser

gly ala pro\*

gly glu asp

gly arg pro\*

gly pro pro\*

gly pro gln

gly ala arg

gly gln pro\*

gly val met

gly phe pro\*

gly pro lys\*

gly ala asn

gly glu pro\*

gly lys ala

gly glu lys\*

gly leu pro\*

gly ala pro\*

gly pro ala  
gly glu glu  
gly lys arg  
gly ala arg  
gly glu pro\*

gly PRO THR  
gly LEU PRO\*

gly pro pro\*

gly glu arg

gly GLY pro\*

gly SER arg

gly phe pro\*

gly ALA asp

gly VAL ala

gly pro lys\*

gly PRO ALA

gly glu arg

gly ALA PRO\*

gly PRO ALA

gly pro lys\*

gly SER PRO\*

gly GLU ALA

gly arg pro\*

gly glu ALA

gly leu pro\*

gly ala LYS\*

gly leu thr

gly SER pro\*

gly SER PRO\*

gly pro ASP

gly lys THR

gly pro PRO\*

gly PRO ALA

gly GLN ASN

gly arg pro\*

gly pro pro\*

gly pro PRO\*

gly ala arg

gly gln ALA

gly val met

gly phe pro\*

gly pro LYS

gly ala ALA

gly glu pro\*

gly lys ala

gly glu ARG

gly VAL pro\*

gly PRO pro\*

402

432

462

492

522

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 gly ala glu.  
 gly pro pro  
 gly pro ala  
 gly pro ala  
 gly glu arg  
 gly glu gln  
 gly ala pro  
 gly pro ser  
 gly phe gln  
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 gly pro pro  
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 gly ser pro  
 gly ala gln  
 gly leu gln  
 gly pro arg  
 gly leu pro  
 gly thr pro  
 gly thr asp  
 gly pro lys  
 gly ala ser  
 gly pro ala  
 gly pro pro  
 gly ala gln  
 gly pro pro  
 gly leu gln  
 gly met pro  
 gly glu arg  
 gly ala ala  
 gly ile ala  
 gly pro lys  
 gly asp arg  
 gly asp val  
 gly glu lys  
 gly pro glu

552

582

612

gly thr asp  
 gly pro lys\*  
 gly ala ALA  
 gly pro ala

gly ASN asp  
 gly ALA lys\*  
 gly ASP ALA  
 gly ALA PRO\*

642

gly ile ala  
 gly pro lys\*  
 gly asp arg  
 gly asp val  
 gly glu lys  
 gly pro glu

gly LEU PRO\*  
 gly pro LYS  
 gly asp arg  
 gly asp ALA  
 gly PRO lys  
 gly ALA ASP

672

gly ala pro  
 gly lys asp  
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 gly leu thr  
 gly pro ile  
 gly pro pro  
 gly pro ala  
 gly ala asn  
 gly glu lys  
 gly glu val  
 gly pro pro  
 gly pro ala  
 gly ser ala  
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 gly ala pro  
 gly glu arg  
 gly glu thr  
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 gly pro pro  
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 gly phe pro  
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 gly arg val  
 gly pro pro  
 gly ser asn  
 gly asn pro  
 gly pro pro  
 gly pro pro  
 gly pro ser

gly ala pro

gly ala pro

gly ASP VAL

gly glu lys\*  
 gly glu val  
 gly pro pro\*

gly ALA PRO\*  
 gly ASP LYS  
 gly glu ALA  
 gly pro SER

702

732

gly gln pro  
 gly ala lys\*  
 gly GLY gln  
 gly glu ala  
 gly gln lys\*  
 gly asp ala  
 gly ala pro\*

gly gln PRO\*  
 gly ala LYS  
 gly GLU PRO\*  
 gly ASP ala  
 gly ALA lys\*

gly asp ala  
 gly ala pro\*

762

792

822

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gly pro lys  
gly ala arg  
gly asp ser  
gly pro pro  
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gly glu pro  
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gly glu lys  
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gly pro ser  
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gly pro pro  
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gly ser pro  
gly ala asp  
gly pro pro  
gly arg asp  
gly ala ala  
gly val lys  
gly asp arg  
gly glu thr  
gly ala val  
gly ala pro  
gly ala pro

852

882

912

942

972



gly pro pro  
gly ser pro  
gly pro ala  
gly pro thr  
gly lys gln  
gly asp arg  
gly glu ala  
gly ala gln  
gly pro met  
gly pro ser  
gly pro ala  
gly ala arg  
gly ile gln  
gly pro gln  
gly pro arg  
gly asp lys  
gly glu ala  
gly glu pro  
gly glu arg  
gly leu lys  
gly his arg  
gly phe thr  
gly leu gln  
gly leu pro  
gly pro pro  
gly pro ser  
gly asp gln  
gly ala ser  
gly pro ala  
gly pro ser  
gly pro arg  
gly pro pro  
gly pro val  
gly pro ser  
gly lys asp  
gly ala asn  
gly ile pro  
gly pro ile  
gly pro pro  
gly pro arg  
gly arg ser  
gly glu thr  
gly pro ala  
gly pro pro  
gly asn pro  
gly pro pro  
gly pro pro  
gly pro pro  
gly pro gly

1002

1032

1062

1092

1119

WHAT IS CLAIMED:

1           1. A method for treating an autoimmune disease in a  
2 mammal, the method comprising administering to said mammal an  
3 effective amount for treating said disease of a bystander  
4 antigen, said antigen eliciting the release of transforming  
5 growth factor beta (TGF- $\beta$ ) at a locus within the body of said  
6 mammal wherein T cells contributing to autoimmune response are  
7 found to suppress the T-cells contributing to said response.

1           2. The method of claim 1 wherein said bystander  
2 antigen is specific to an organ or tissue afflicted by immune  
3 attack during said disease.

1           3. The method of claim 2 wherein said bystander  
2 antigen is not an autoantigen.

1           4. The method of claim 2 wherein said bystander  
2 antigen is an autoantigen.

1           5. The method of claim 2 wherein said bystander  
2 antigen comprises a portion of an autoantigen but excludes at  
3 least one epitope of said autoantigen that is recognized by  
4 immune system cells contributing to said disease.

1           6. The method of claim 1 wherein said bystander is  
2 administered to said mammal via oral route.

1           7. The method of claim 1 wherein said bystander is  
2 administered to said mammal via inhalation.

1           8. The method of claim 1 wherein:  
2               said bystander antigen is administered by oral  
3 route or by inhalation;  
4               said oral or inhalable bystander antigen elicits  
5 suppressor T-cells that cause the release of TGF- $\beta$ ;

6                   said bystander antigen is not specific to an organ  
7 or tissue afflicted by immune attack during said disease;  
8                   said method further comprising also administering  
9 to said mammal the same bystander antigen via parenteral route,  
10 thereby causing said suppressor T-cells to be targeted to the  
11 same loci within the body of said mammal wherein the cells  
12 contributing to autoimmune attack are found.

1                   9. The method of claim 1 wherein said disease is  
2 selected from the group of multiple sclerosis and animal models  
3 therefor, and said bystander antigen is selected from the group  
4 of myelin basic protein, proteolipid protein, fragments thereof  
5 comprising at least one suppressive epitope, and combinations of  
6 any two of the foregoing.

1                   10. The method of claim 9 wherein said bystander  
2 antigen comprises MBP peptide 21-40.

1                   11. The method of claim 1 wherein said disease is  
2 selected from the group consisting of rheumatoid arthritis and  
3 animal models therefor and said bystander antigen is selected  
4 from the group consisting of Type I collagen, Type II collagen,  
5 fragments thereof comprising a suppressive epitope and  
6 combinations of two or more of the foregoing.

1                   12. The method of claim 1 wherein said disease is  
2 selected from the group consisting of Type I diabetes and animal  
3 models therefor and said bystander antigen is selected from the  
4 group consisting of glucagon, insulin, fragments thereof  
5 comprising at least one suppressive epitope, and combinations of  
6 two or more of the foregoing.

1                   13. The method of claim 1 wherein said disease is  
2 selected from the group consisting of uveoretinitis and animal  
3 models therefor and said bystander antigen is selected from the

4 group consisting of S-antigen, interphotoreceptor retinoid  
5 binding protein (IRBP), fragments thereof comprising at least one  
6 suppressive epitope, and combinations of two or more of the  
7 foregoing.

1 14. The method of claim 1 further comprising  
2 administering to said mammal an amount of a synergist effective  
3 in combination with said bystander antigen to treat said disease.

1 15. A pharmaceutical oral dosage form for treating an  
2 autoimmune disease in a mammal, the form comprising:

3 an effective amount for treating said disease of  
4 a bystander antigen, said antigen upon administration eliciting  
5 the release of transforming growth factor beta (TGF- $\beta$ ) at a locus  
6 within the body of said mammal wherein T cells contributing to  
7 autoimmune response are found to suppress the T-cells  
8 contributing to said response; and

9 a pharmaceutically acceptable carrier or diluent.

1 16. The oral dosage form of claim 15 wherein said  
2 bystander antigen is specific to an organ or tissue afflicted by  
3 immune attack during said disease.

1 17. The oral dosage form of claim 16 wherein said  
2 bystander antigen is not an autoantigen.

1 18. The oral dosage form of claim 16 wherein said  
2 bystander antigen is an autoantigen.

1 19. The oral dosage form of claim 16 wherein said  
2 bystander antigen comprises a portion of an autoantigen  
3 comprising an immunosuppressive epitope but excludes at least one  
4 epitope of said autoantigen that is recognized by immune system  
5 cells contributing to said disease.

1           20. The oral dosage form of claim 15 wherein said  
2 disease is selected from the group of multiple sclerosis and  
3 animal models therefor, and said bystander antigen is selected  
4 from the group of myelin basic protein, proteolipid protein,  
5 fragments thereof comprising at least one suppressive epitope,  
6 and combinations of any two of the foregoing.

1           21. The oral dosage form of claim 20 wherein said  
2 bystander antigen comprises MBP peptide 21-40.

1           22. The oral dosage form of claim 15 wherein said  
2 disease is selected from the group consisting of rheumatoid  
3 arthritis and animal models therefor and said bystander antigen  
4 is selected from the group consisting of Type-I collagen, Type II  
5 collagen, fragments thereof comprising a suppressive epitope and  
6 combinations of two or more of the foregoing.

1           23. The oral dosage form of claim 15 wherein said  
2 disease is selected from the group consisting of Type I diabetes  
3 and animal models therefor and said bystander antigen is selected  
4 from the group consisting of glucagon, insulin, fragments thereof  
5 comprising at least one suppressive epitope, and combinations of  
6 two or more of the foregoing.

1           24. The oral dosage form of claim 15 wherein said  
2 disease is selected from the group consisting of uveoretinitis  
3 and animal models therefor and said bystander antigen is selected  
4 from the group consisting of S-antigen, interphotoreceptor  
5 retinoid binding protein (IRBP), fragments thereof comprising at  
6 least one suppressive epitope, and combinations of two or more of  
7 the foregoing.

1           25. The oral dosage form of claim 15 further  
2 comprising administering to said mammal an amount of a synergist

3 effective in combination with said bystander antigen to treat  
4 said disease.

1           26. A pharmaceutical inhalable dosage form for  
2 treating an autoimmune disease in a mammal, the form comprising:  
3           an effective amount for treating said disease of  
4 a bystander antigen, said antigen upon administration eliciting  
5 the release of transforming growth factor beta (TGF- $\beta$ ) at a locus  
6 within the body of said mammal wherein T cells contributing to  
7 autoimmune response are found to suppress the T-cells  
8 contributing to said response; and  
9           a pharmaceutically acceptable carrier or diluent.

1           27. The inhalable dosage form of claim 26 wherein said  
2 bystander antigen is specific to an organ or tissue afflicted by  
3 immune attack during said disease.

1           28. The inhalable dosage form of claim 26 wherein said  
2 bystander antigen is not an autoantigen.

1           29. The inhalable dosage form of claim 26 wherein said  
2 bystander antigen is an autoantigen.

1           30. The inhalable dosage form of claim 26 wherein said  
2 bystander antigen comprises a portion of an autoantigen  
3 comprising an immunosuppressive epitope but excludes at least one  
4 epitope of said autoantigen that is recognized by immune system  
5 cells contributing to said disease.

1           31. The inhalable dosage form of claim 26 wherein said  
2 disease is selected from the group of multiple sclerosis and  
3 animal models therefor, and said bystander antigen is selected  
4 from the group of myelin basic protein, proteolipid protein,  
5 fragments thereof comprising at least one suppressive epitope,  
6 and combinations of any two of the foregoing.

1                   32. The inhalable dosage form of claim 31 wherein said  
2 bystander antigen comprises MBP peptide 21-40.

1                   33. The inhalable dosage form of claim 26 wherein said  
2 disease is selected from the group consisting of rheumatoid  
3 arthritis and animal models therefor and said bystander antigen  
4 is selected from the group consisting of Type I collagen, Type II  
5 collagen, fragments thereof comprising a suppressive epitope and  
6 combinations of two or more of the foregoing.

1                   34. The inhalable dosage form of claim 26 wherein said  
2 disease is selected from the group consisting of Type I diabetes  
3 and animal models therefor and said bystander antigen is selected  
4 from the group consisting of glucagon, insulin, fragments thereof  
5 comprising at least one suppressive epitope, and combinations of  
6 two or more of the foregoing.

1                   35. The inhalable dosage form of claim 26 wherein said  
2 disease is selected from the group consisting of uveoretinitis  
3 and animal models therefor and said bystander antigen is selected  
4 from the group consisting of S-antigen, interphotoreceptor  
5 retinoid binding protein (IRBP), fragments thereof comprising at  
6 least one suppressive epitope, and combinations of two or more of  
7 the foregoing.

1                   36. The inhalable dosage form of claim 26 further  
2 comprising administering to said mammal an amount of a synergist  
3 effective in combination with said bystander antigen to treat  
4 said disease.

Figure 1

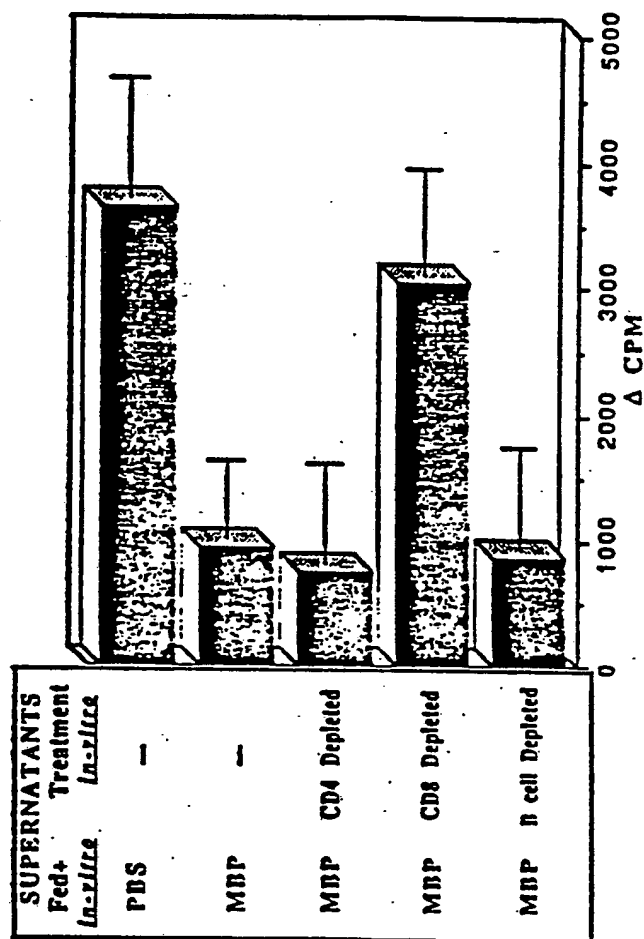




Figure 2

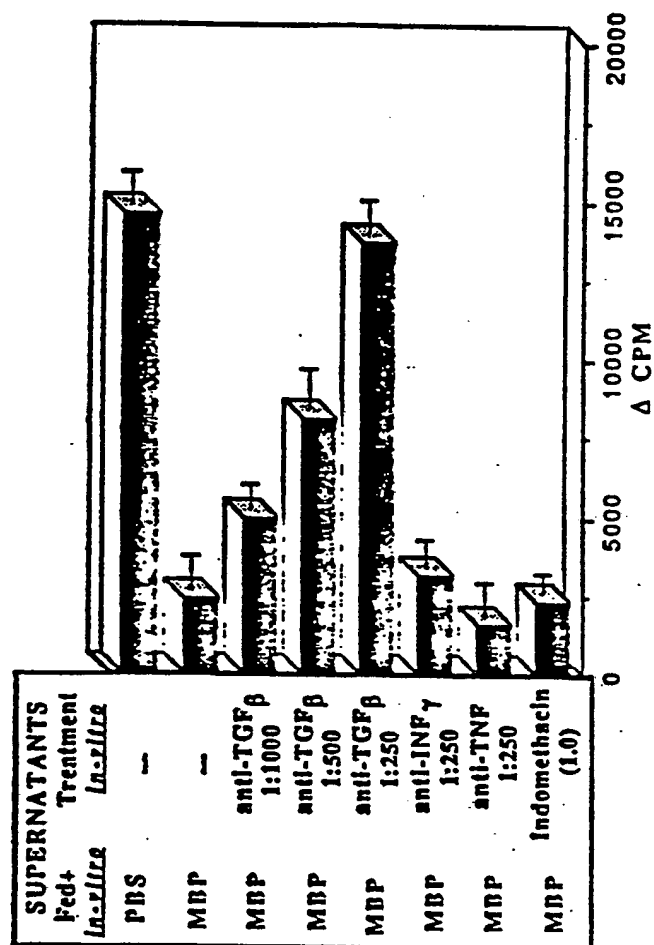


Figure 3

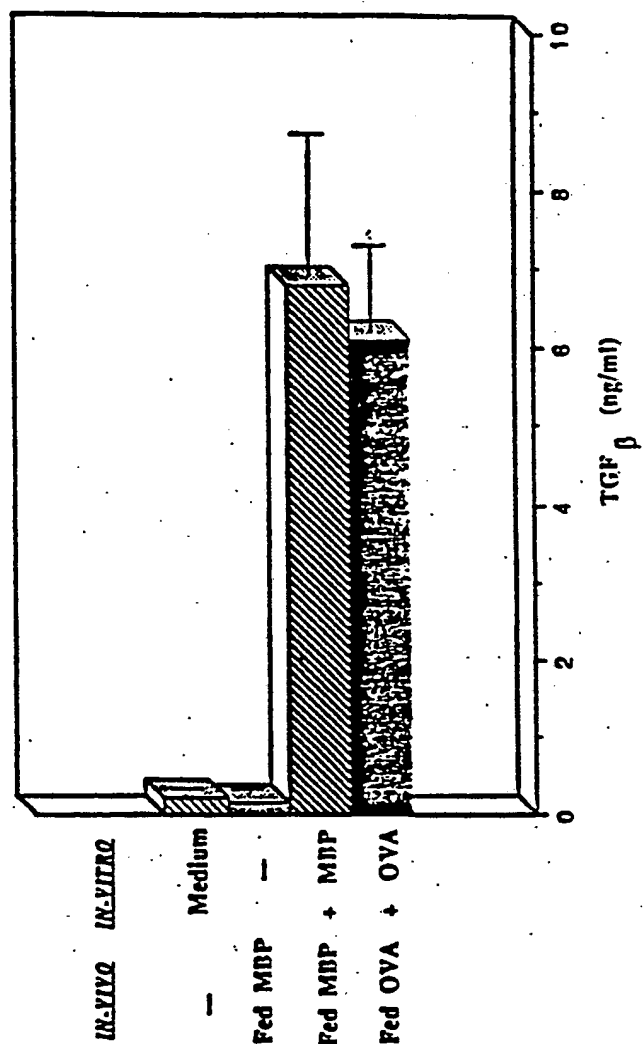


Figure 4

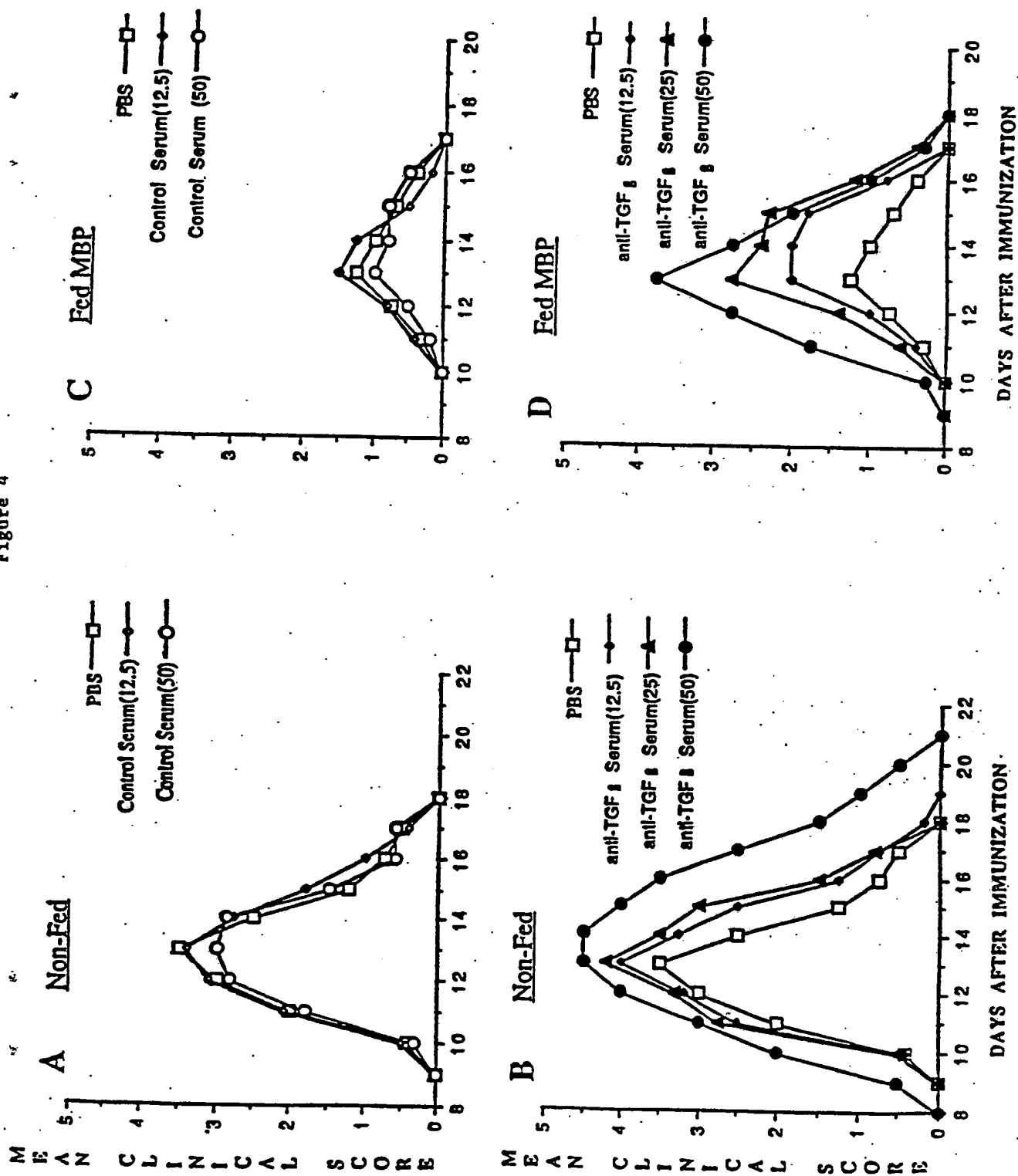
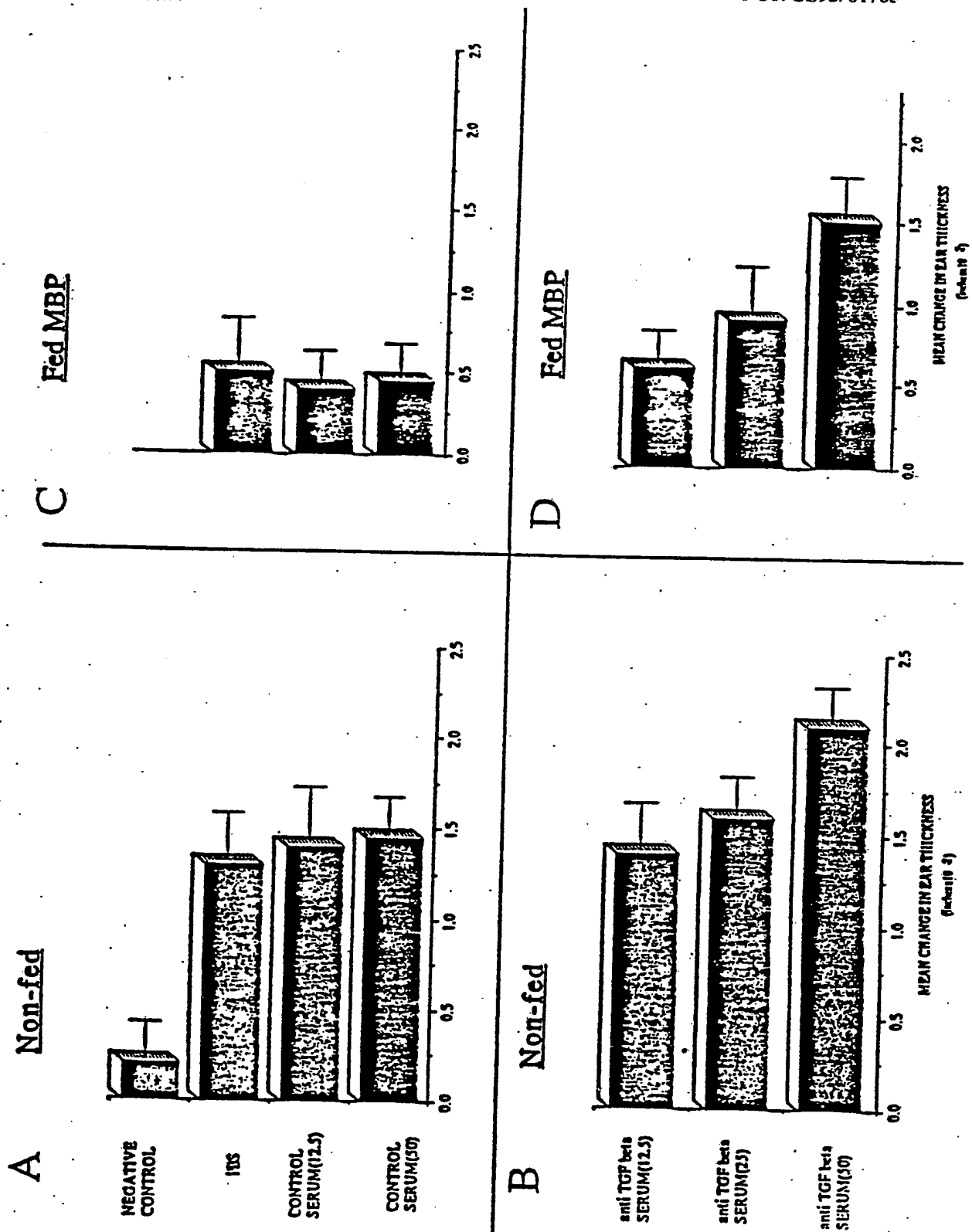


FIGURE 3



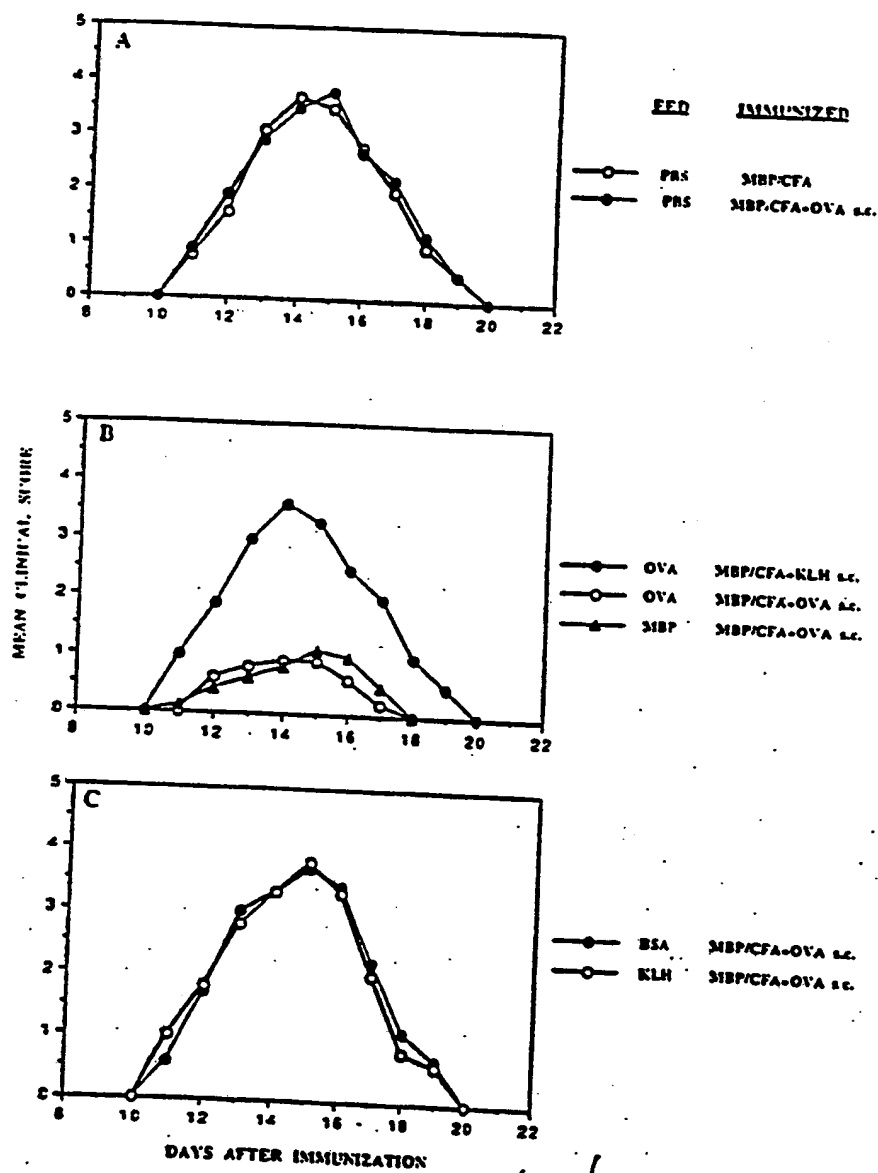
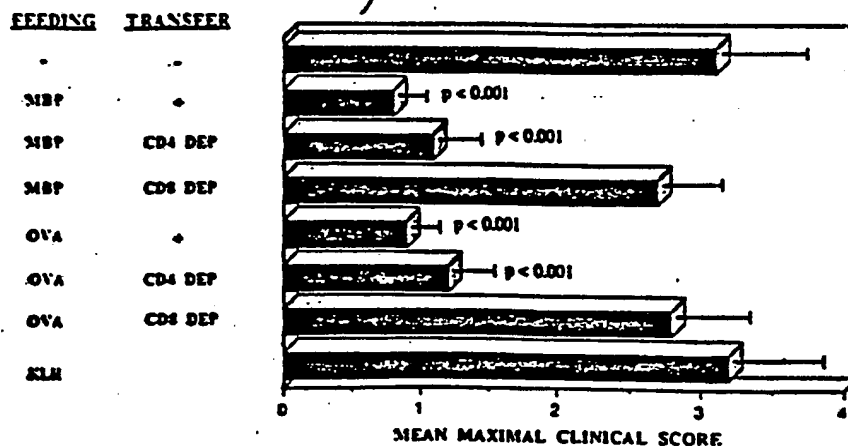
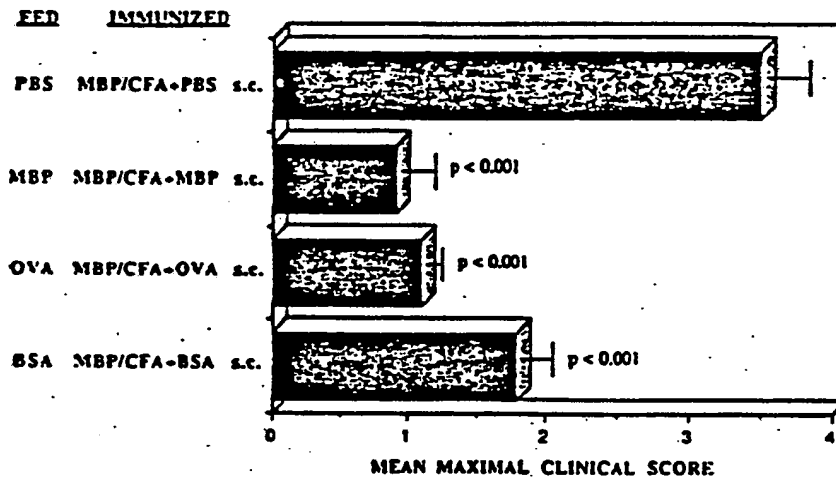
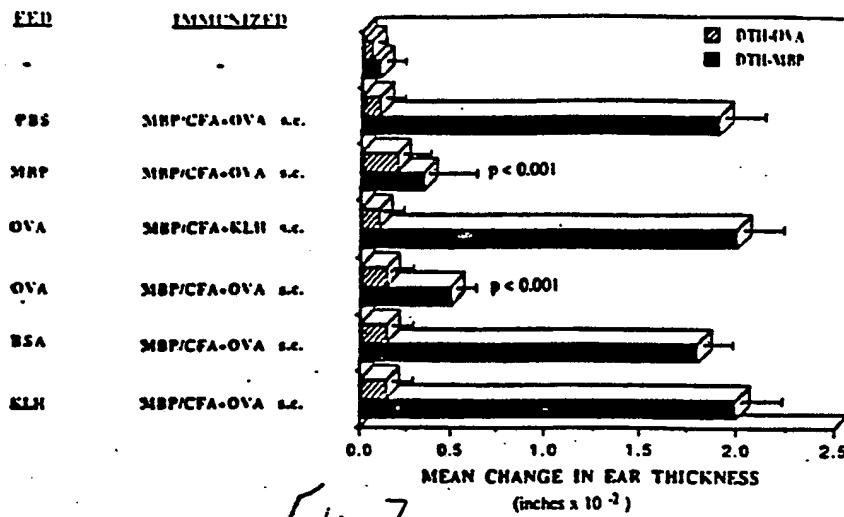


Fig. 6



## MBP-Peptides triggering Lewis rat MBP-primed lymphnode cells

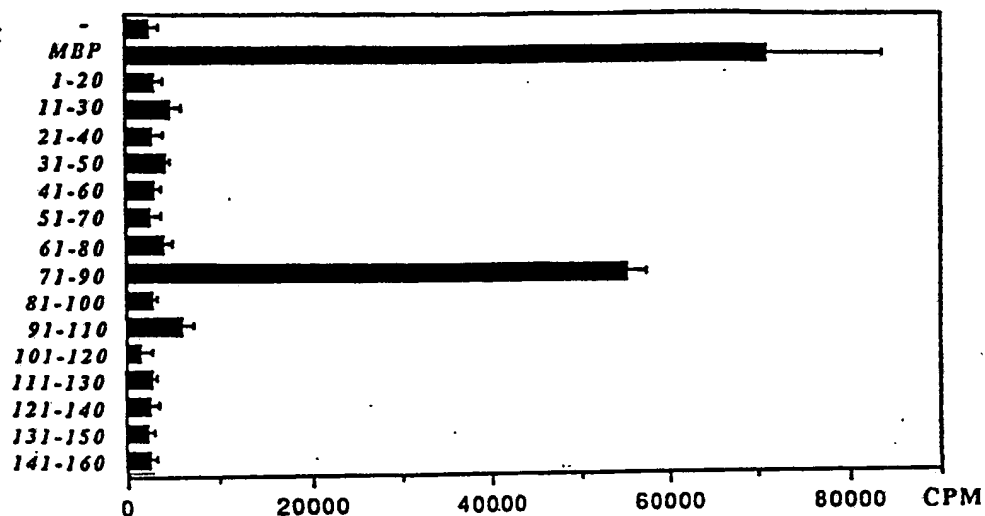


Fig 10

## MBP-Peptides triggering MBP-fed spleen cells to Suppress OVA-line in TW system

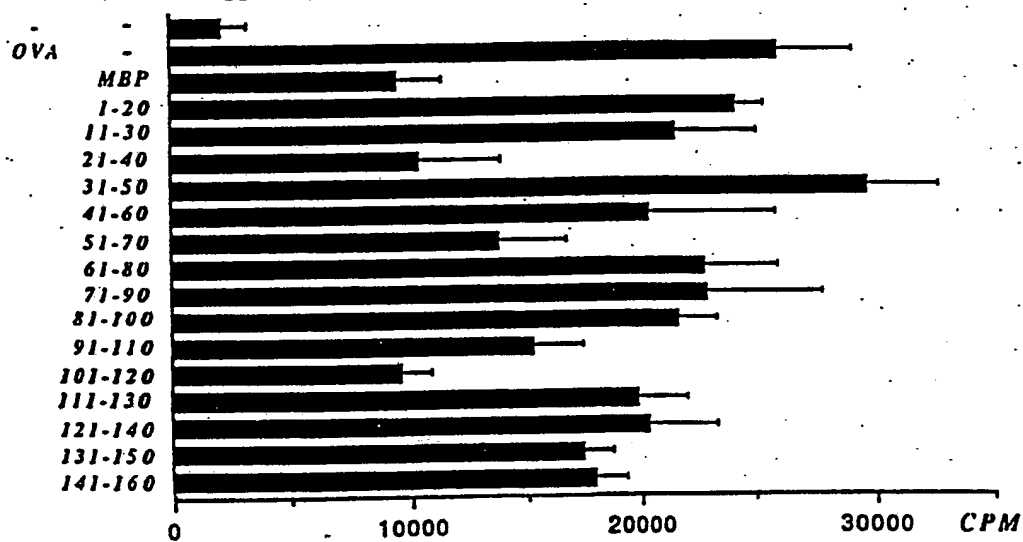
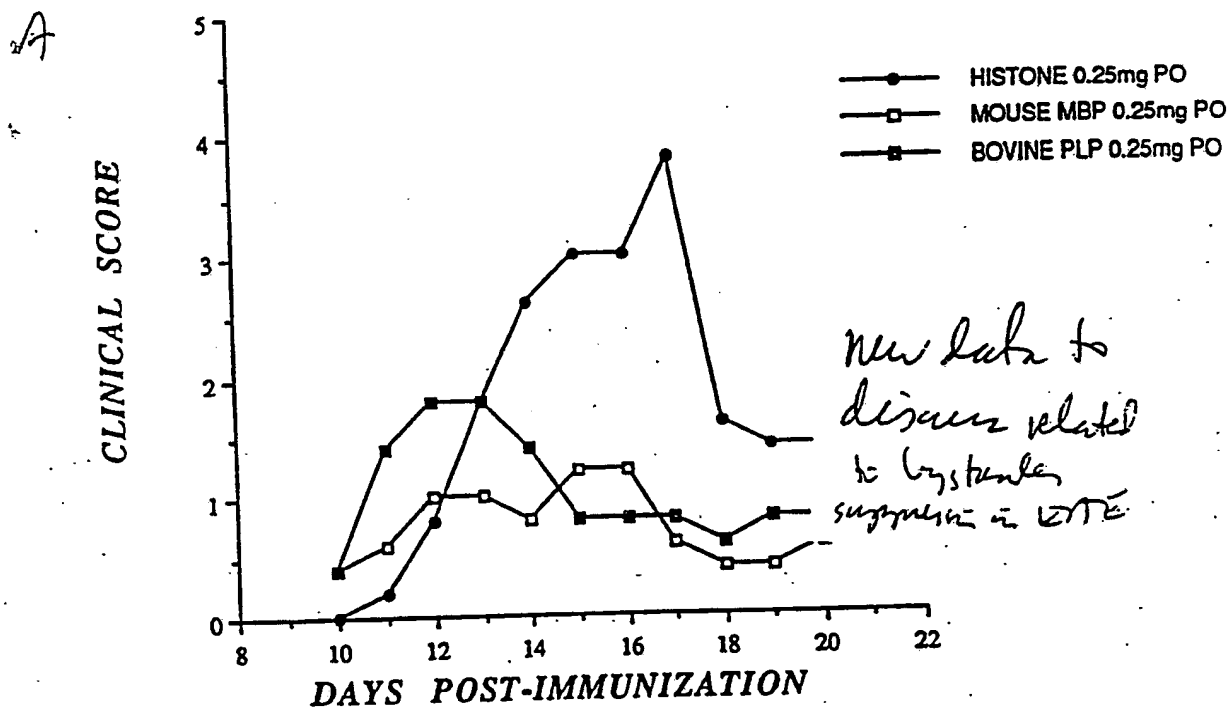


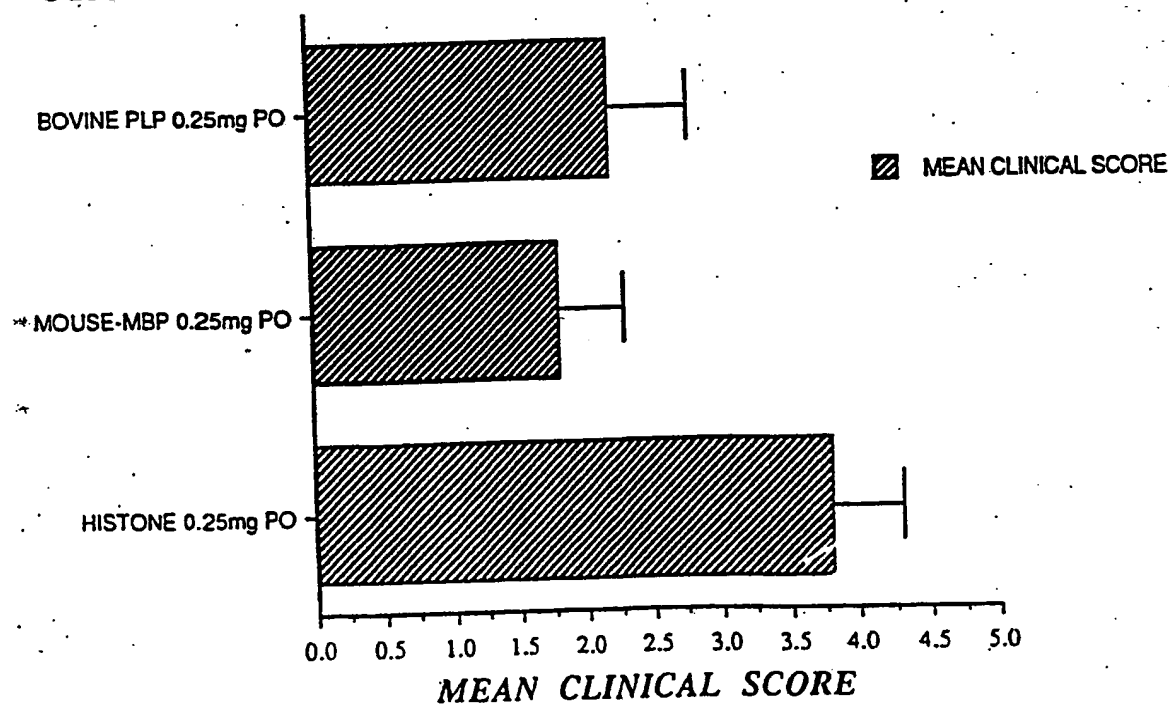
Fig 11

9/13  
fl. 12

CLINICAL DATA FROM SJL/J MICE EAE STUDY  
#33/N8 (1/29/92), IMMUNIZED WITH PLP (140-160)/CFA ON DAY 0&7.



## GROUP





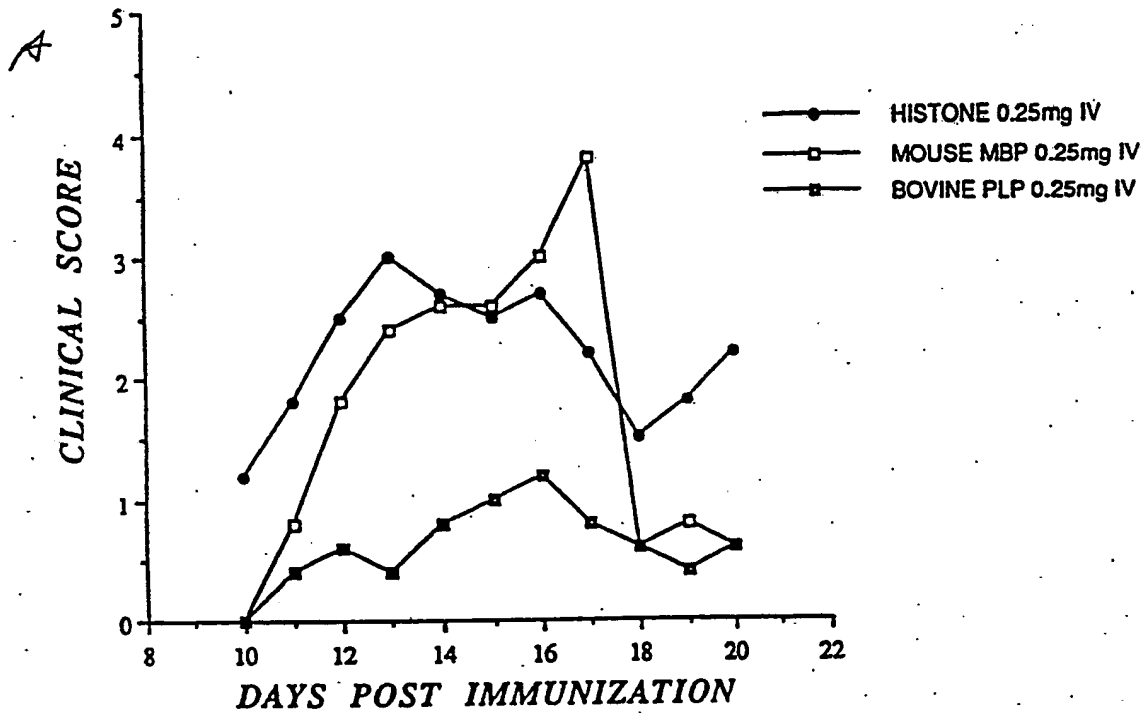
10/13

WO 93/16724

PCT/US93/01705

Fig 13

NICAL DATA FROM SJL/J MICE EAE STUDY  
N8 (1/29/92) , IMMUNIZED WITH PLP(140-160)/CFA ON DAY 0&7.



### GROUP

BOVINE PLP 0.25mg IV

MOUSE MBP 0.25mg IV

HISTONE 0.25mg IV

■ MEAN CLINICAL SCORE

Suppression of ETE induced by 71-90  
by feeding various peptides

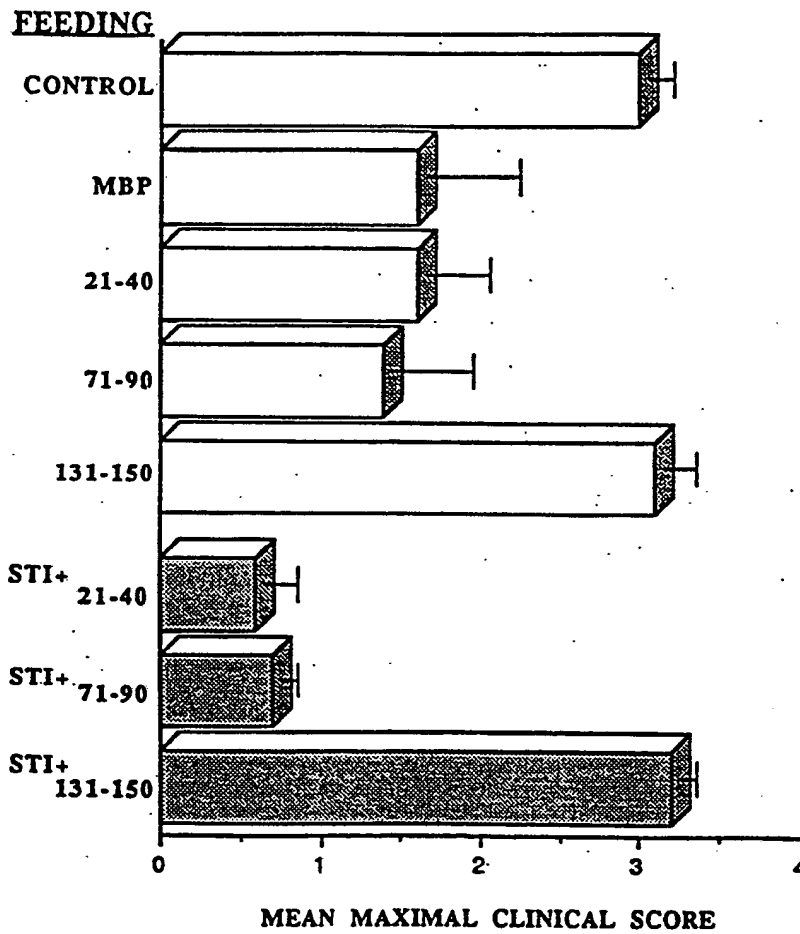
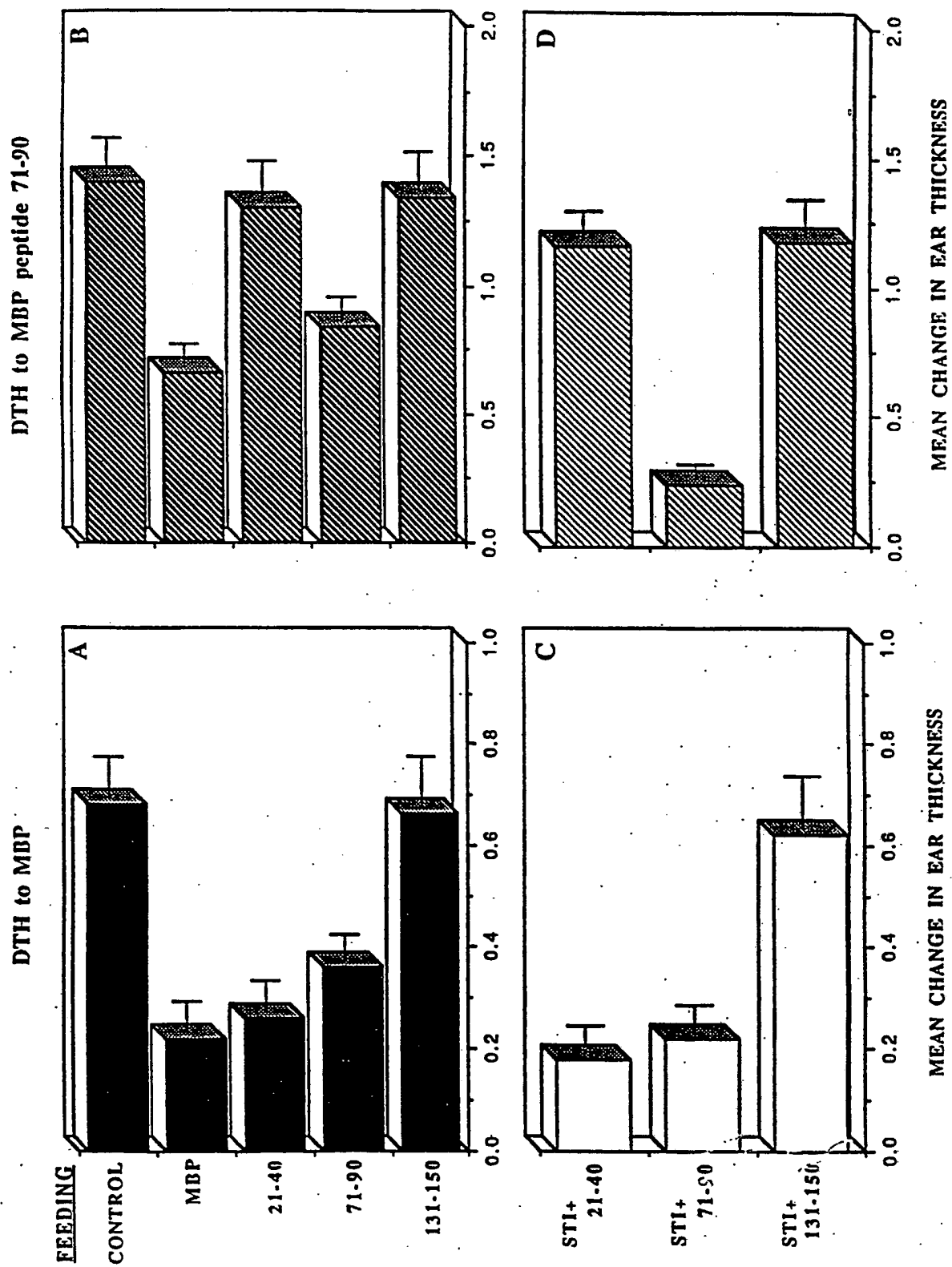
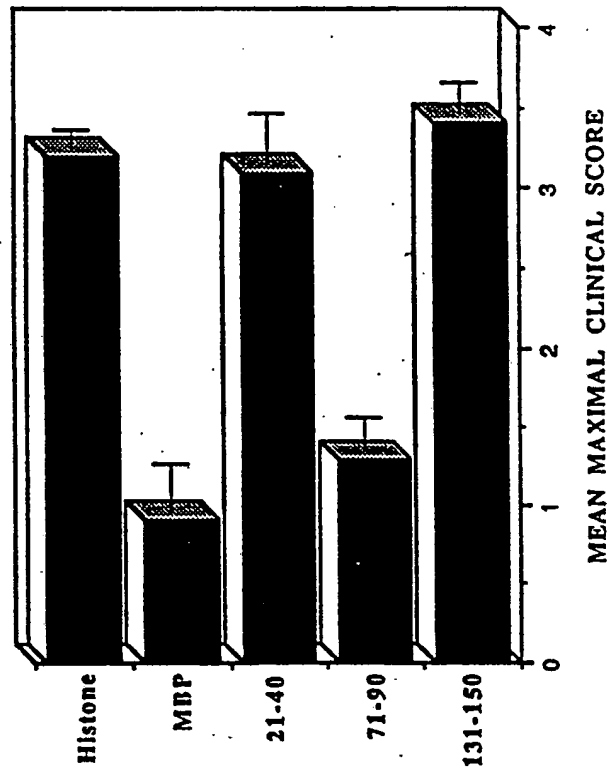


Fig 14



Suppression of EAE by i.v. tolerization with MBP and MBP-peptides  
prior to disease expression(days 8' and, 9' post immunization)



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/01705

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 39/00, 37/02; C07K 3/00; A61K 37/12, 37/24.  
US CL : 424/88; 530/303, 308, 324, 350, 356, 399.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88; 530/303, 308, 324, 350, 356, 399.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	The Journal of Immunology, Volume 121, No. 6, issued December 1978, Richman et al. "Enterically Induced Immunologic Tolerance: Induction of Suppressor T Lymphocytes by Intragastric Administration of Soluble Proteins", pages 2429-2434, especially page 2429 and Figure 1.	1-36
Y	The Journal of Immunology, Volume 140, No. 2, issued 15 January 1988, Higgins et al, "Suppression of Experimental Autoimmune Encephalomyelitis by Oral Administration of Myelin Basic Proteins and Its Fragment", pages 440-445, especially page 440, Tables 1 and 2.	1-10, 14-21, 25-32 and 36



Further documents are listed in the continuation of Box C.



See patent family annex.

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* E		earlier document published on or after the international filing date
* L		document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* O		document referring to an oral disclosure, use, exhibition or other means
* P		document published prior to the international filing date but later than the priority date claimed
	* X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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	* Z	document member of the same patent family

Date of the actual completion of the international search

22 APRIL 1993

Date of mailing of the international search report

20 APR 1993

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/01705

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Abstract, 6th International Congress of Immunology, Toronto, Canada, issued July 1986, Whitacre et al, "Suppression of Experimental Allergic Encephalomyelitis (EAE) by the Oral Administration of Myelin Basic Protein (MBP)", Reference No. 3.62.21.	1-10, 14-21, 25-32 and 36
Y	Proc. Natl. Acad. Sci. USA, Volume 83, issued October 1986, Nagler-Anderson et al, "Suppression of Type II Collagen-induced Arthritis by Intragastric Administration of Soluble Type II Collagen", pages 7443-7446.	1-8, 11, 14-19, 22, 25-30, 33 and 36

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/01705

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS and Medline.

Search terms: Autoimmune disease?, transforming growth factor beta (TGF $\beta$ ), bystander antigen?, autoantigen#, collagen, insulin, retinoid binding proteins, myelin basic proteins, oral or inhalation administration.

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